

1-1-2012

Identification and characterization of novel $\alpha 1 \text{IbB3}$ -integrin binding proteins in human platelets

Rand Alhashmi

Royal College of Surgeons in Ireland

Citation

Alhashmi R. Identification and characterization of novel $\alpha 1 \text{IbB3}$ -integrin binding proteins in human platelets [MSc Thesis]. Dublin: Royal College of Surgeons in Ireland; 2012.

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Identification and Characterization of Novel $\alpha_{IIb}\beta_3$ -Integrin Binding Proteins in Human Platelets

A thesis submitted to the National University of Ireland for the Degree of Master in Science

Rand Alhashmi B.Pharm

Under the supervision of Dr. Sarah O'Neill PhD and Professor Niamh Moran

Department of Molecular and Cellular Therapeutics

Royal College of Surgeons in Ireland

123 St. Stephens Green

Dublin 2

Submitted October 2011

Declaration

I hereby declare that all the work prepared in this thesis was carried out by me. To the best of my knowledge, the results presented in this thesis originated from the present study except where references have been made.

Signed: _____

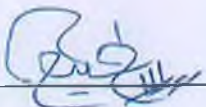
Student number: 04234162

Date: _____

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Signed:

A handwritten signature in blue ink, appearing to be 'B. S.', written over a horizontal line.

Student number: 04234162

Date:

23/5/12

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Abbreviations

ACD: Acid Citrate Dextrose

ADP: adenosine diphosphate

APS: Ammonium Persulphate

CaCl₂: Calcium Chloride

cAMP: Cyclic Adenosine 3'-5' MonoPhosphate

CHO: Chinese Hamster Ovary

Co-IP: Co-immunoprecipitation

dH₂O: Deionised Water

DTT: Dithiothreitol

FITC: Fluorescent isothiocyanide

HRP: Horse Radish Peroxidase

IP: Immunoprecipitation

LC/ MS: Liquid chromatography/ Mass spectrometry

Pal-FF: Palmitylated-KVGFFKR peptide

PE: Phycoerythrin

PG: Prostaglandin

PGE-1: Prostaglandin E₁

PINCH-1: Particularly interesting new Cys-His protein 1

PKAR1 α : cAMP dependent PKA1 α regulatory subunit

PMSF: Phenyl Methyl Sulphonyl Fluoride

PPI: Protein Protein Interaction

PRP: Plasma Rich Platelets

R.T: Room Temperature

S.B: Sample Buffer

SCBT: Santa Cruz Bio Technology

SDS: Sodium Dodecyl Sulphate

TRAP: Thrombin Receptor Activating Peptide

TBS: Tris Buffered Saline

TBST: Tris Buffered Saline Tween

WP: Washed Platelets

Abstract

The pathological role of platelets in cardiovascular disease is well established. The major platelet cell adhesion molecule, $\alpha_{IIb}\beta_3$ has been a target for research into anti-thrombotic agents. It is a cell-surface cell adhesion molecule belonging to the integrin family and it plays a critical role in platelet function. It is present on the plasma membrane of circulating platelets in an inactive or quiescent state. Following vascular injury or thrombotic triggers, it 'activates' by undergoing a profound change of conformation. However, little is known about the molecular mechanisms underlying this activation. Previous work has identified a regulatory sequence (KVGFFKR) within the cytoplasmic tail that controls integrin activation (Stephens et al., 1998). Subsequently a number of novel platelet cytoplasmic proteins were discovered that interact with this regulatory region. They include CIB1 (Naik et al., 1997), AUP-1 (Kato et al., 2002), PP1 (Vijayan et al., 2004), PP2A (Gushiken et al., 2008), ICln and RN181 proteins (Larkin et al., 2004). Recent work by, confirmed all these proteins and also identified a number of novel platelet proteins that may regulate integrin function by competing for binding to the α -integrin regulatory region (Raab et al., 2010). The aims of this study are to characterize some of these proteins and to investigate their capacity to control integrin function in human platelets.

The proteins that were chosen for this study are cyclic AMP protein kinase α (regulatory subunit; PKAR1 α) and 2 members of LIM protein family: PINCH-1 and the paxillin homologue Hic-5.

Western blotting was used to verify the presence of these proteins in human platelets and to examine their capacity to be altered by platelet activation. We demonstrated that platelets express all 3 proteins. In addition, I show evidence for activation-dependent modification of these proteins in response to pro-thrombotic stimuli. PKAR1 α and Hic-5 show evidence of post-translational modification, whereas PINCH-1 shows evidence of proteolytic degradation in response to platelet activation. Our preliminary evidence (Raab et al., 2010) suggested binding of PKAR1 α , PINCH-1 and Hic-5 to the integrin $\alpha_{IIb}\beta_3$ via a motif in the platelet membrane. However not all those findings were consistent with the results that we got. By using co-immunoprecipitation we found that the proteins PKAR1 α and PINCH-1 do not bind with the integrin directly. On the other hand, our observations for Hic-5 suggest that it may be a novel integrin regulating protein.

Acknowledgements

I would like to thank my dear country United Arab Emirates for giving me such an opportunity to further carry on my post graduate study. Our kind rulers Sheikh Khalifa Bin Zayed AlNhayen, Sheikh Mohammed Bin Rashed AlMaktoom and Sheikh Sultan Bin Mohammed AlQasimi.

Two years of research in Laboratory 4 were full of enjoyment, cooperation and encouragement. Emily, Desmond, Federica, Kalyan, Annaciara, Tadhg and Vincent I was lucky to have nice colleagues in the lab with me. Special thanks to Seamus Allen for his continuous support.

Niamh, my great supervisor, understanding, supportive, helpful and generous even through her busy days in work.

Sarah is so thoughtful and kind with open doors and heart to listen to you any time. Thank you for your encouragement. I'm happy that I did my master research with such a brilliant supervisors Niamh and Sarah.

Least but not last, I'm very grateful to my beloved family for their support during my years of study.

Abstracts and Communications

Identification and characterisation of novel $\alpha\text{IIb}\beta 3$ -integrin binding proteins in human platelets

Rand Alhashmi, Niamh Moran and Sarah O'Neill. Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland.

Presented as a poster at RCSI Research Day April 2011 and RCSI Pharmacy Conference April 2011

Chapter 1

General Introduction

Platelets are anucleate blood cells that have discoid shape and a life-span of 7-9 days. They perform a regulatory role in haemostasis and are targets for drug action in cardiovascular or thrombotic disease. Platelets become activated when they adhere to damaged blood vessels. During activation they release biomolecules from either the dense or alpha-granules, such as adenosine diphosphate (ADP), calcium and fibrinogen. In addition, platelet activation results in the activation of the platelet-specific receptor $\alpha_{IIb}\beta_3$ that belongs to the integrin family. Integrins are cysteine-rich heterodimeric molecules that alter their affinity for ligands in response to cellular activation (O'Neill et al., 2000). The integrin $\alpha_{IIb}\beta_3$ is an abundant platelet cell-surface adhesion molecule and plays a critical role in platelet function. However, the exact intracellular mechanism of integrin activation is not fully understood. The integrin $\alpha_{IIb}\beta_3$ is present on the plasma membrane of circulating platelets in an inactive or quiescent state (Hynes, 2002). The integrin $\alpha_{IIb}\beta_3$ (also termed GpIIb/IIIa) is composed of two separate protein subunits, the alpha (α) subunit α_{IIb} and the beta (β) subunit β_3 . The α -subunit is made of a heavy and a light chain that are connected by a disulphide bonds. It has a conserved motif $^{989}\text{KxGFFKR}^{995}$ in the cytoplasmic region that was characterized in a previous study (Stephens et al., 1998). A cell-permeable peptide corresponding to this motif activates integrins in platelets, suggesting that this motif plays a pivotal role in integrin activation (Stephens et al., 1998).

Several studies that were conducted in our laboratory investigated the interaction of cytoplasmic proteins with the platelet integrin $\alpha_{IIb}\beta_3$ in the expectation that a molecular mechanism for integrin activation would emerge. We hypothesise that the integrin tail of α_{IIb} regulates the signalling cascade in platelet by modulating the interaction of cytoplasmic and cytoskeletal proteins. To know more about the proteins and interactions, a study was designed to explore the nature of platelet proteins that bound to the integrin cytoplasmic tail in resting and activated platelets (Daxecker et al., 2008); (Raab et al., 2010). Three scientific methods were used in these studies to detect and characterise new proteins: (1) peptide affinity chromatography using the conserved $^{989}\text{KVGFFKR}^{995}$ motif, (2) LC-MS/MS to identify bound platelet proteins and (3) protein chips to detect novel proteins that had an affinity for KVGFFKR using information obtained from protein:protein interaction (PPI) databases.

In the studies by Raab and Daxecker, they immobilized the critical, conserved $^{989}\text{KVGFFKR}^{995}$ peptide onto an inert bead that could then be used as an affinity matrix, as illustrated in Figure 1.1A-C. Platelet lysates prepared from resting or activated platelets were

allowed to interact with the column before being eluted with Guanidinium HCl (Figure 1.1D). The eluted peaks were separated on SDS-PAGE (Figure 1.1E) and excised for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The eluted proteins were identified from their mass spectra using the GPM (Global Proteome Machine) online resource.

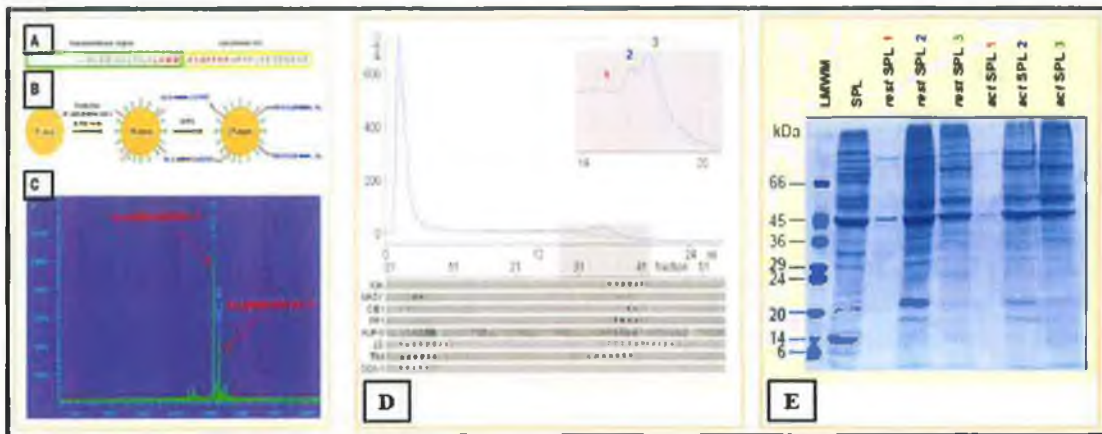


Figure 1.1: A-C show the sequence of synthesising the peptide affinity column. D is a chromatogram of human platelet lysate with proteins that bind to the KVGFFKR motif which are retained by the peptide affinity column. Below the chromatogram, sample dot-bots for the presence of ICln, RN181 (previously known as MASY), CIB-1, PPI, AUP-1, integrin β_3 , TIM and COX1 are shown. E is an SDS-PAGE analysis of proteins from resting or activated sonicated platelet lysates (SPL) that were separated on the peptide affinity chromatography. Peaks 1, 2 and 3 correspond to the observed peaks eluted from the column and labelled in the chromatogram in D (Raab et al., 2010).

It was discovered that all previously identified integrin-binding proteins including CIB1 (Naik et al., 1997), AUP-1 (Kato et al., 2002), PP1 (Vijayan et al., 2004), PP2A (Gushiken et al., 2008), ICln and RN181 proteins (Larkin et al., 2004) bound to the integrin α_{IIb} cytoplasmic motif as outlined in Table 1.1. 38 additional platelet proteins were bound to the peptide affinity chromatography column and identified by LC-MS/MS, but it is not certain if these are specific interactors or non-specific interactors. Some proteins were abundant in the platelet lysate but not in the peptide column eluate (such as Vinculin). Other proteins were the opposite i.e. abundant in the column eluate but not in the platelet lysate (Raab et al., 2010).

These proteins are candidates as novel integrin-regulating proteins.

Proteins enriched on peptide column	KVGFFKR Affinity Extracts		Platelet lysates 1-10	EF
	Resting	Activated		
HIC-5/ ARA55 Q43294				100
actin-like protein O17421				81
TUBULIN BETA-5				81
Latent-TGF beta-binding protein Q14766				49
Placental ribonuclease inhibitor				49
Tripeptidyl-peptidase 1 Q14773				49
Calcium and integrin-binding protein 1Q99828				36
14-3-3 protein epsilonP62298				36
Dynactin subunit 3 mplex subunit 22 Q75935				36
Alpha-actinin-2 P35609				25
Myosin light chain 9B UniProtKB/P14640				25
Ubiquitin carboxyl-terminal hydrolase 5 P46974				25
Cathepsin A				18
cAMP-dependentPKA1 P10644				25
Filamin- A Q75369				14
Tropomyosin P07951				14
Ser/Thr phosphatase 2A catalytic P62714				11
HEAT SHOCK-RELATED 70 KDA PROTEIN				11
G- protein G beta subunit 2 P62879				11
Ras-related protein Rab-6A 40				9
Ser/Thr phosphatase PP1-gamma cat P36873				9
G- protein Galpha P50148				5
Myosin regulatory light chain MRLC3 P19105				5
Tubulin alpha-2 Q13748				5
Actin-related protein 2/3 complex 41kDa Q15143				5
ATP synthase subunit alpha P25705				4
T-complex protein 1 thetaP50990				4
Talin-2 Q9Y406				4
WD repeat protein 1 Q75083				3
Bridging integrator 2 ei-associate Q9UBW5				3
Beta-parvinQ9HBI1				3
PINCH-1 P49059				2
Talin-1 Q9Y400				2
Multimerin-1 Q13201				2
Integrin-linked protein kinase Q13418				2

Table 1.1: Proteins Enriched in Peptide Column

38 proteins were identified in eluates from Resting or Activated platelets which bound to the KVGFFKR-peptide column. The data shown is peptide values (color coded from yellow (low) to red (high) depending on protein abundance) from 5 experiments for resting platelets, 4 separate experiments for activated platelets and 10 separate platelet lysates. In some cases, these proteins were low abundance proteins which were not observed in platelet lysates (Black). The enrichment ratio was calculated as described in the methods section. The known integrin binding proteins CIB1 and protein phosphatases are color coded yellow. The proteins selected for this study are highlighted in orange.

They include PKAR1 α , PINCH-1 and Hic-5. These three proteins were chosen for further study because they represent three different levels of enrichment and abundance: Hic-5 is the most highly enriched of all the proteins observed in our experiment. In fact, it is higher than the previously identified integrin-binding proteins CIB-1 and protein phosphatases 1 & 2. PKAR1 α appears to be enriched in activated platelets, even though it is only observed in 3 out of 9 of our affinity experiment. Neither Hic-5 nor PKAR1 α are ever observed in platelet lysates, suggesting that these are low-abundance proteins. Finally, PINCH-1 is selected, because it is always observed at higher intensity in affinity extracts than in lysates. It is an

abundant platelet protein that is usually observed in a complex with Parvin and integrin linked kinase (ILK). However, despite it being an abundant protein in platelet lysates, we considered it interesting because it was always enriched in our affinity extracts. In addition, Parvin and integrin linked kinase, its co-associated proteins, were both also enriched in our samples.

The MS analysis techniques that were used have a percentage of error associated with them. Thus proteins might be observed in the affinity extracts even if they do not regulate integrin function. This may be because they are abundant proteins and are difficult to remove from our samples. Alternatively, they may be 'sticky' proteins with high levels of charge and/or hydrophobicity permitting them to adhere to complementary regions on our peptide affinity column. Finally, proteins may bind indirectly to the integrin regulatory region, via another directly-binding protein. We have represented this argument in Figure 1.2 below.

We believe that the proteins exist in platelets in four different states relative to integrin function. A protein that adheres to the integrin in the resting platelet state is termed 'X'. A protein that adheres to the integrin in the activated platelets only is termed 'Y'. A protein that binds to the integrin regardless of its activation status is called 'Z'. Finally, the last protein is one that does not bind to the integrin at all and is referred to as 'W'. In this thesis, I will attempt to define which category PKAR1 α , PINCH-1 and Hic-5 proteins belong to.

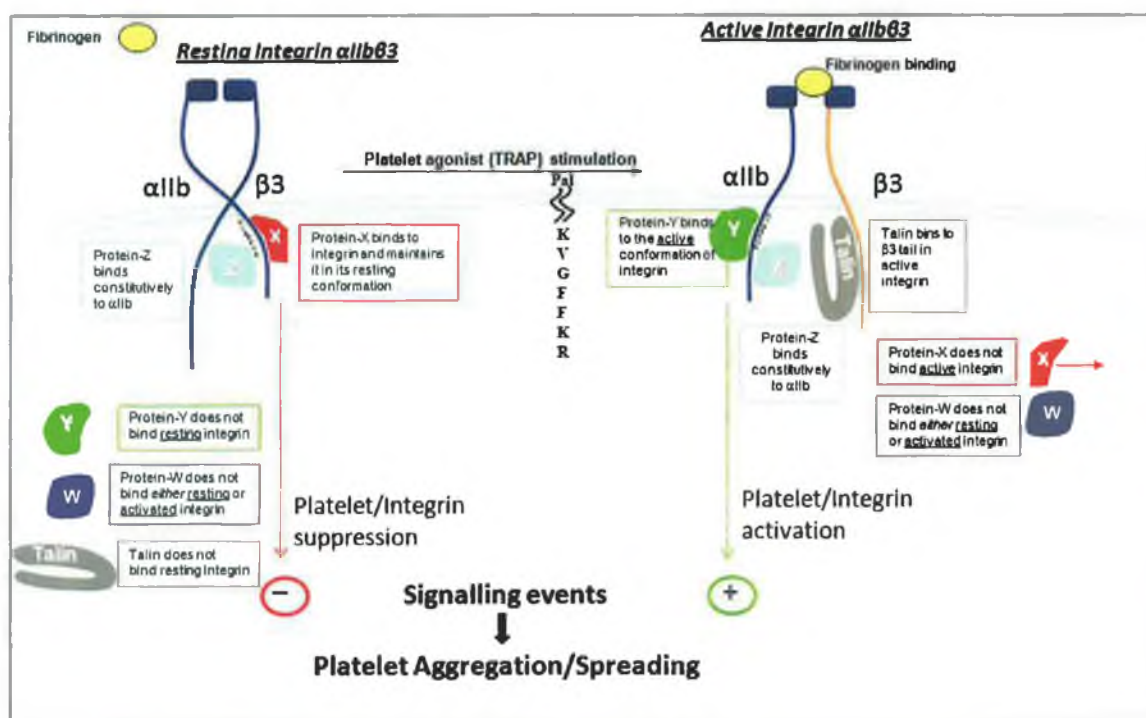


Figure 1.2: Model of protein binding to integrin cytoplasmic tail

In order to confirm the MS data and confirm if the observed binding is significant, our study aims to verify that the proteins PKAR1 α , PINCH-1 and Hic-5 are present in platelets using western blotting technique. Then we will verify whether the proteins are associated directly with the integrin $\alpha_{IIb}\beta_3$ by using co-immunoprecipitation technique. Finally, we will determine for each protein which definition they conform to in the X, Y, Z and W definition above. To date, little is known about PKAR1 α and PINCH-1 in platelets. On the contrary, Hic-5 has been mentioned in the literature but its role in integrin regulation is uncertain.

cAMP-dependent PKAR1 α

The second messenger cAMP controls inside-out signaling pathways such as regulating metabolism and proliferation. cAMP-dependent protein kinase-A (PKA) holoenzyme has a tetrameric structure. It has two regulatory (R) and two catalytic (C) subunits (Bossis and Stratakis, 2004). cAMP levels are elevated by inhibitors of platelet activation such as the endothelial-derived prostaglandin (PG) I₂. PG I₂ is released constantly by intact endothelial cells and interacts with PG I₂ receptors on the platelet surface. On activation of these receptors, platelets respond by elevating intracellular cAMP levels. This inhibits or modulates the ability of the platelets to activate.

In the presence of cAMP, the holoenzyme of PKA breaks down into a dimer of regulatory subunits and 2 free catalytic subunits. Both subunits have multiple isoforms: α , β and γ of types I and II. PKAR1 α enzyme corresponds to the regulatory subunit (R) of type 1 α . There are four types of the regulatory subunit R1 α , R1 β , R2 α and R2 β . The isozymes (α and β) of regulatory subunit 1 of protein Kinase A exist in human neoplastic B cell line (Reh) (Tasken et al., 1993). PKAR1 α was first discovered in platelets by our research group (Raab et al., 2010). The regulatory subunit is the major component of type I of PKA that controls serine-threonine kinase activity (Bossis and Stratakis, 2004).

PINCH-1 (LIMS1)

This particularly interesting new Cys-His protein 1 (PINCH-1) is also referred to as LIMS1. PINCH-1 is a cytoplasmic protein that is composed of 5 LIM domains. LIM domain is a double-zinc finger motif unit that is found in many proteins (Dawid et al., 1998). Theoretically, PINCH-1 binds to Hic-5 (TGFB1I1) by the LIM zinc-binding 5 domain (The Universal Protein Resource). PINCH-1 has a role in cell differentiation and proliferation. It also forms a complex with integrin-linked kinase (ILK) and Parvin (Stanchi et al., 2009). PINCH1-ILK-Parvin molecules create a complex called IPP. Both PINCH-1 and ILK are important in forming this complex and the removal of either of these for any reason affects the down regulation of the proteins (Stanchi et al., 2009). In the Raab study, all 3 components of this complex were observed to be enriched in the affinity column eluates, suggesting that this complex binds to the platelet alpha integrin cytoplasmic tail (Raab et al., 2010). The intensity of binding for each individual protein was always greater in resting than in activated platelets. We therefore decided to study PINCH-1 protein because it forms an important complex with ILK and Parvin and may play a role in integrin activation.

Hic-5 (TGFB1I1)

Hic-5 is the only member of paxillin family in human platelets (Osada et al., 2001). It is a cytoskeletal protein with 4 LIM domains at the carboxy terminal (Hagmann et al., 1998). TGFB1I1 is the abbreviation of the genetic name: transforming growth factor beta-1-induced transcript 1 (The Universal Protein Resource). It regulates protein interactions and presents in the focal adhesion points in the cells. Hic-5 in activated platelets is tyrosine phosphorylated and negatively regulates platelets aggregation (Rathore et al., 2007). Hic-5 binds to different α integrin tails and regulates their function (Liu et al., 1999). However, (Liu et al., 1999) also

showed that Hic-5 protein does not appear to bind to α_{IIb} . In contrast, however, it was found by our group that Hic-5 binds to the integrin chain- α_{IIb} regulatory peptide in a chromatography column (Raab et al., 2010). We therefore studied the role of Hic-5 as a potential integrin-regulatory protein in activated platelets.

The future plan is to find out the precise molecular effects of the novel proteins on the integrin's cytoplasmic domain in order to better understand the choreography of signalling proteins that co-ordinate integrin activation in response to prothrombotic signals.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Equipments and Reagents

All chemicals and products were obtained at highest grade possible and were from Sigma-Aldrich (UK), except where specifically mentioned below. Thrombin receptor activating peptide (TRAP: SFLLRN) was obtained from Bachem Distribution Services GMBH-Germany. Palmitylated KVGFFKR peptide was custom synthesized by JPT Peptide Technologies, Germany. Octyl- α/β -Glucoside was obtained from Fluka Analytical-Switzerland, HaltTM Protease Inhibitor Cocktail and Phosphatase Inhibitors were from Thermo Scientific, (Rockford, U.S.A.). Protein G-Sepharose 4 fast was from GE Healthcare, UK. SuperSignal® West Pico Chemilumiscent Substrate is obtained from Thermo Scientific, UK.

2.1.2 Antibodies

2.1.2.1 Primary Antibodies

The following primary antibodies were obtained from Santa Cruz Bio-Technology (SCBT) company, Inc.-(Germany) except where specifically mentioned below.

Table 2.1.

Target Protein	Source	Supplier's SCBT (code)	Details	Dilution for WB	Predicted Molecular weight of the protein
Anti-PKAR1 α	Mouse monoclonal	3546C2a	Raised against recombinant protein corresponding to a region near N-terminus of PKA1 α regulatory of human origin	1: 100 - 1:1000	48kDa

Anti-PKAR1 α	Goat polyclonal	C-14	Affinity purified antibody against a peptide mapping within an internal region of PKA1 α regulatory of human origin	1:200	48kDa
Anti-PINCH-1	Goat polyclonal	D-18	Affinity purified antibody against a peptide mapping within an internal region of human origin	1:200 – 1:1000	37kDa
anti-Hic-5	Mouse monoclonal	F6	Raised against amino acids 1-75 mapping at the N-terminus of human Hic-5 origin	1:100 – 1:500	55kDa
anti-Hic-5	Rabbit polyclonal	H75	Raised against amino acids 1-75 mapping at the N-terminus of Hic-5 of human origin	1:200	55kDa
anti-Hic-5	Mouse monoclonal	Clone 34 (#611165)	Mouse IgG ₁ , from BD Transduction Laboratories	1:500	55kDa
anti-Integrin α IIb (SZ.22)	Mouse monoclonal	SZ.22	Raised against washed human platelets. Initial batches from SCBT; subsequent batches from Beckman Coulter-Co Clare-Ireland. At 128kDa	1:200 - 1:10,000	137kDa
anti-GPIIb (CD42b)	Goat polyclonal	C-20	Raised against a peptide mapping at the C-terminus of human GPIIb	1:500	143kDa

2.1.2.2 Control IgG Antibodies

Antibodies that have been used as controls in immunoprecipitation are normal mouse IgG: sc-2025 and normal rabbit IgG: sc-2027.

2.1.2.3 Secondary Antibodies

Rabbit anti-goat IgG-HRP (SC-2922) was obtained from SCBT. Goat anti-rabbit IgG-HRP (#31460) was obtained from Pierce Immunopure®, Thermo Scientific. Goat anti-mouse IgG₁ was obtained from Beckman Coulter-USA. Secondary antibody dilutions were varied according to optimised experiments and are indicated in Figure legends.

2.2 Methods

2.2.1 Washed platelets preparation

Venous blood was drawn from healthy volunteers who had not taken aspirin or other non-steroidal anti-inflammatory drugs for the preceding 10 days, according to approved protocol by the Royal College of Surgeons in Ireland Research Ethics Committee. Washed platelets were prepared as described (Stephens et al., 1998). Briefly, this involved the anti-coagulation of blood with 15% (v/v) acid-citrate-dextrose (ACD) (38mM citric acid anhydrous, 75mM sodium citrate, 124mM dextrose). Blood was centrifuged at 150 x g for 10 minutes and the platelet rich plasma (PRP), the top fraction, was transferred to a separate tube using a plastic transfer pipette. PRP was acidified with ACD to pH 6.5 and 1 μ M prostaglandin E₁ (PGE₁) was added. The platelets were pelleted at 720 x g for 10 minutes and resuspended in JNL buffer (6mM dextrose, 130mM NaCl, 9mM Na Bicarbonate, 10mM Na Citrate, 10mM Tris, 3mM KCl, 0.81mM KH₂PO₄, 0.9mM MgCl₂·6H₂O pH 7.35). The platelet count was adjusted to 3x10⁵ platelets/ μ l. Washed platelets (WP) were diluted either to 6x10⁵/ μ l for western blotting or higher for co-immunoprecipitation and were supplemented with 1.8mM CaCl₂ immediately prior to use. Platelet count was estimated by analyzing samples on a Sysmex automatic blood analyzer, (Sysmex KX Series, SYSMEX UK LTD-Milton Keynes, MK8 8DF, England, United Kingdom).

2.2.2 Platelet aggregation

Platelet Aggregation was performed at 37°C in a Platelet Aggregation Profiler (BioData PAP-4) (Horsham-USA). Thrombin receptor activating peptide (TRAP) was used as an agonist for platelet aggregation. Pal-KVGFFKR was also used to activate washed platelets. Washed platelets were equilibrated in the aggregometer at 37°C before the addition of agonists. Platelet aggregations were followed for three minutes. Three washed platelet samples were prepared for each western blotting analysis. The first sample was stirred for 3 minutes in the aggregometer in the absence of an agonist as a negative control. This sample is referred to as “resting platelets”. The second sample was treated with TRAP at a final concentration of 10µM, to maximally stimulate platelet aggregation. The third sample was partially activated by the integrin-derived palmitylated peptide at a concentration of 100µM. After 3 minutes in the aggregometer, the samples were lysed on ice using a 10x lysis buffer (0.5mM Octyl- α / β -Glucoside, 10mM PMSF, 20mM Tris pH 7.4, 0.1% Triton-X-100, 50mM EDTA, 5X Halt™ Protease Inhibitor Cocktail and 10X Phosphatase Inhibitors in JNL buffer) to dissolve platelets membrane and stop any protease activities in the platelets. Samples were vortexed every 10 minutes for an hour to ensure full solubilization of all protein in the sample, and then spun to precipitate any insoluble residues. Samples are either frozen at -80°C until required for analysis, or were used immediately. A Bradford assay was used to determine the protein concentration in each sample.

2.2.3 Bradford Assay

Platelet lysate samples are analyzed for protein concentration using the DC Protein Assay from Bio-Rad Corporation. 5µl of samples were loaded in triplets into a 96 well plate that can be read by Wallac plate reader (WallacVictor²™) 1420 Multilabel Counter. Serial dilutions of bovine serum albumin (BSA) in 1X lysis buffer were prepared to generate a BSA standard curve from 0 – 1.5mg/ml. Reagents A' and B were added to the samples in the wells according to the manufacturer's instructions. After an incubation of 15 minutes the absorbance of the samples in the plate were read at 750nm according to the manufacturer's instructions. The concentration of protein in the platelet lysate samples could then be deduced from the standard curve.

2.2.4 Samples preparation for immuno-detection

Frozen human platelet lysates samples were thawed in an ice-bath and mixed with 2X sample buffer at a ratio of 1:1.

2X Sample Buffer (pH 6.8) is composed of: 0.5M Tris base, 4% SDS, 75mM DTT, 2.17M Glycerol, 0.01% Bromphenol Blue. Samples were heated at 95°C for 3 minutes and spun for 1 minute in a mini Spectrafuge that was purchased from Labnet International, Inc. to precipitate any impurities before loading onto SDS-poly-acrylamide gels. In initial experiments, lysates were divided into 2 groups; the first group contains three previously prepared samples that are treated with a reducing sample buffer containing DTT, and the other group is treated with a 2X non-reducing sample buffer pH 6.8 that does not contain DTT (0.5M Tris base, 4% SDS, 2.17M Glycerol, 0.01% Bromphenol Blue). In the case of immunoprecipitation experiments, samples were treated with only reducing sample buffer.

2.2.5 Immunoprecipitation

Two protocols were used to optimise the experimental conditions and both gave same results. The first protocol that was used is from SCBT Company and the second is from the University of Melbourne (UM) for immunoprecipitation of platelet proteins.

2.2.5.1 Immunoprecipitation according to method A (SCBT)

Protein G-sepharose beads were used when immunoprecipitating with monoclonal antibody. The beads were washed 3 times from slurry with ice-cold high-salt (hs) TBST (150mM NaCl, 20mM Tris, pH 7.4, 0.1% Tween 20) and incubated rotating for 30 minutes at 4°C with 1ml (~600x10³/ul) platelet lysates and 0.25ug/ml of IgG-control antibody from the same species of the primary antibody. The precleared lysate mix was spun and the supernatant retained for the next step. The pelleted beads were washed 5 times with ice-cold TBST (hs) 1X, and eluted with 2X sample buffer. This sample is called the 'negative control' or IgG sample. All steps were performed at 4°C. The supernatant was transferred to a fresh eppendorf and incubated with the required immunoprecipitation antibody (0.2-2ug/ml) for 1-2 hours rotating at 4°C. Freshly washed beads of approximately (25ul/lane) were then added to the supernatant/antibody mix and incubated over night rotating at 4°C. The next day the beads were pelleted and washed 5 times with ice-cold hs TBST and eluted with 2X sample buffer. This sample is called the 'IP sample' and represents the specific immunoprecipitated fraction.

2.2.5.2 Immunoprecipitation according to Method B (UM)

Protein A-Agarose beads were used to immunoprecipitate with polyclonal antibodies. The beads were washed 3 times from slurry with ice-cold PBS and incubated rotating for 1 hour at 4°C with 0.5ml (~600x10³/ul) platelet lysates for preclearing. The platelet lysate was microfuged briefly to pellet the beads (72g for 30 seconds). The supernatant was divided into two and removed to fresh eppendorfs. The first eppendorf is the negative control which is incubated with an IgG-antibody and the second is for the pull-down which is incubated with the immunoprecipitation antibody. Both antibodies added for incubation of equal amounts (5ug/500ul), rotating at 4°C from 2 hours to overnight. Then equal amounts (25ul/lane) of fresh washed beads were added to both eppendorfs and kept rotating for 1-2 hours at 4°C. After that, the beads were pelleted gently and washed 3 times with PBS, eluted with 2X sample buffer, boiled and spun. Samples were ready for use.

2.2.6 Immunoblotting

SDS-PAGE: Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis was performed according to standard procedures (Laemmli, 1970). 10% (#25221) and 4-20% gradient (#25224) Precise protein gels were obtained commercially from Fisher Thermo Scientific (PIERCE)-U.K. Prepared samples were loaded onto the 10% or gradient gels. Samples were run at a constant voltage as follows: 60V for 20 minutes then 100V for 1 hour, in a 1X running buffer (25mM Tris, 192mM Glycine, 0.1% SDS). After that the gel was finished running, a mini Trans-Blot Cell was assembled by placing the gel against a polyvinylidene fluoride (PVDF, transferring membrane that is immersed for 3 minutes in methanol before putting it in the sandwich) between nylon pads and blotting papers to form a sandwich (the nylon pads, blotting papers and transferring membrane were soaked in a transferring buffer prior to preparing the sandwich). Transfer is achieved by applying a constant voltage (100V) to drive the proteins from the gel onto the PVDF in a BIO-RAD blotting tank for 1 hour. For this purpose, a 1X transfer buffer is prepared from a 10X stock (250mM Tris, 1.92M Glycine, 0.2% SDS). One volume of 10x transferring buffer is diluted with 2 volumes of methanol in dH₂O. After transfer, the membrane was removed from the sandwich and is blocked with 5% of either BSA (Bovine serum albumin) or 5% milk powder in 0.1% Tween20 in TBS (TBST: 20mM Tris Base, 137mM NaCl pH7.4) for one hour at room temperature. Three washes for 10 minutes for each blot were done using TBST. The blots were then incubated over night with a primary antibody at 4°C at the dilution indicated in the Table 1 above. The next morning after

washing the blots three times for 10 minutes for each blot, they were incubated with a secondary antibody for 1- 2 hours rotating at room temperature. A number of different secondary antibody dilutions were used during the course of western blotting. The dilutions that were routinely used are 1:50,000 for anti-mouse, 1:10,000 for anti- goat and for anti-rabbit. The membranes were washed again three times for 10 minutes each using 1X TBST. Membranes were exposed to SuperSignal® West Pico Chemiluminescent Substrate for 5 minutes to detect the antibodies. The blots were visualised by either UVP WorkLS software or by developing images using Kodak Biomax XAR film that was obtained from Sigma Aldrich IRL in the dark room. Each western blot was performed in pairs: one blot was incubated with the immunoprecipitating antibody α IIb (SZ.22) as a control and the second was incubated with one of the primary antibodies against the novel proteins. Often, the blots were reprobed with the opposing antibody to verify results. At least 4 separate western blots on separate donors were performed for each antibody, as indicated in the Figure legends.

2.2.7 Normalising Bands

The band's intensity was determined using UVP imaging system (Vision WORKsLS) and ImageJ software for all western blots. The bands intensity was normalized to all samples' bands in the experiment. Thus the total intensity of the western blot bands was summed for the 9 lanes on each gel. This was assigned a value of 100. Each band was expressed as a percentage of this total. In this way, the variance between experiments was minimized.

Chapter 3

Optimizing Western Blotting Conditions

The aim of this chapter is to optimize the experimental conditions for western blot analysis and to discuss the characterization of the proteins of interest. In this study we focused on PKAR1 α , PINCH-1 and Hic-5 proteins in platelet lysates. In order to identify if there are any protein modifications upon cellular activation, we sought to identify suitable antibodies to detect these proteins. Throughout this chapter, problems arising during western blotting will be discussed.

3.1 Introduction

The basic theory of western blotting technique is that cellular proteins can be separated by SDS-PAGE and transferred to a nitrocellulose or PVDF membrane that can be probed by specific antibodies for the protein of interest. The primary antibody binds specifically to the protein from the platelet lysates on the western blot membrane. The primary antibody is detected by an appropriate secondary antibody that is coupled to a detection system.

Good western blots should reveal a band that shows a strong signal on a clear background. The position of the band should correspond to the predicted molecular weight of the protein of interest by comparison to molecular weight standards. Good protein bands should appear as a straight line. Sometimes a protein band is detected at a molecular weight that is slightly different from the predicted molecular weight for the reasons described below.

There are a number of crucial aspects that can influence the results of western blotting: choosing the correct gel percentage, choosing an appropriate antibody and preparing the buffers at the correct pH. In addition, background staining and non-specific binding can be reduced by inclusion of various reagents in the wash buffers, altering the blocking agent, blocking for longer periods of time and including high-salt or detergents in the wash buffers.

3.1.1 Choosing the right gel percentage

SDS Poly-Acrylamide Gels can be prepared with varying percentages of polymeric acrylamides, usually 5% to 20%. The percentage of the gel should be chosen according to the molecular weight of the protein of interest. The relationship between the molecular weight or size of the protein and the gel percentage is inversely proportional, so that high molecular weight proteins (> 100 kDa) will separate poorly in a high percentage gel (15%) but will separate with greater resolution in a lower percentage gel (7%). Thus low molecular weight proteins resolve better on high percentage gel. SDS is a denaturing detergent that is negatively

charged. When proteins bind to SDS they denature and travel vertically through the gel and separate according to their charge. However, most proteins are charged at the pH of SDS-PAGE running gel, (pH 8.8) and the charge is proportional to their size. Proteins therefore separate in SDS polyacrylamide gels according to their size, from the highest weight to the lowest.

3.1.2 Selecting an Appropriate Antibody

Antibodies are one of the most abundant research tools that are used in analytical methods such as western blotting, co-immunoprecipitation and enzyme-linked immunosorbent assays (ELISA). To have a validated antibody, it must be specific, selective and have reproducible profile for its purpose. The definition of antibody validation by the food and drug agency (FDA) is “the process of demonstrating through the use of specific laboratory investigations, that the performance characteristics of an analytical method are suitable for its intended analytical use”. Therefore, choosing a good antibody can be done either by the selection of a commercial antibody and testing it in the laboratory or by checking previous publications that investigate the protein of interest (Bordeaux et al., 2010). If the antibody is chosen from commercial suppliers without the benefit of support from published literature, then the available datasheets should be examined carefully. If the antibody is chosen from published literature, then it is important to compare initial results with those that are published. Usually, we can expect to observe a single band at a certain molecular weight that corresponds to a single protein in a cellular lysate. However, the presence of a doublet can be due to several reasons such as post-translational modification by phosphorylation or ubiquitination (Raab et al., 2010).

3.2 Results

For the purposes of this study, we investigated the presence of three proteins found in human platelets. The proteins are cAMP-dependent PKA-regulatory subunit (PKAR1 α), particularly interesting new Cys-His protein 1 (PINCH-1) and a paxillin homolog (Hic-5). We sought to examine changes of expression of these proteins upon platelet activation. Therefore, western blotting analysis using specific antibodies was utilised in order to look at their presence in platelets. To date, little is known about PKAR1 α and PINCH-1 proteins particularly in platelets and there are no conclusive publications regarding them. Therefore, the knowledge of appropriate antibodies for use in western blotting was not available in the literature.

However, the presence of Hic-5 in platelets is mentioned in the literature (Hagmann et al., 1998); (Osada et al., 2001). In order to identify suitable antibodies for use in platelet studies, we examined expression of each protein with selected antibodies using western blotting technique. We were able to select antibodies for Hic-5 from the published literature. However, we had to use our instincts for the choice of antibodies for PINCH-1 and PKAR1 α .

Washed platelets samples were used in all western blot experiments. Lysates from three different platelet preparations were prepared as follow: (1) Platelets that were kept quiescent or “resting”; (2) Platelets that were maximally activated using thrombin receptor activating peptide (TRAP) using conditions that are well-established in the literature, and (3) platelets that were partially activated by the integrin-derived palmitylated peptide (KVGFFKR), as previously described by our group (Stephens et al., 1998); (Bernard et al., 2009) . Two sets of conditions were used to prepare the platelet lysates for electrophoresis: reduced and non-reduced; wherein thiol bonds responsible for the protein secondary structure are left intact (non-reduced) or the samples are subjected to a reducing chemical environment with dithiothreitol (DTT).

All primary antibodies were bought from the commercial company SCBT in Germany except where mentioned.

3.2.1 Identification of PKAR1 α in human platelets

Western blot experiments were done to detect the presence of PKAR1 α protein in human platelets. Two different antibody clones were purchased from Santa Cruz Biotechnology (SCBT) (3546C2a: Sc-81641) (PKAR1a, SCBT-b) and (C-14: sc-18800) (PKAR1a, SCBT-a).

The preliminary experiments identified that the first antibody 3546C2a was not adequate in detecting the protein. The bands obtained from the western blots from this antibody were faint and not clear. They were observed as faint bands at approximately 48kDa, as seen in Figure 3.1A. In order to improve detection, we used different series of dilutions of the antibody (Figures 3.1A and 3.1B). In addition, we used two experimental conditions: reduced and non-reduced samples.

In all experiments, the bands detected were weak or absent. Additional changes were done to improve the band's detection such as using higher gel percentage, adjusting antibody's

dilutions and loading higher protein concentration (data not shown). Regardless of all the introduced changes (Figures 3.1A and 3.1B), blots continued to be vague, and non-reproducible leading us to the conclusion that either PKAR1 α is not present in human platelets or that the 3546C2a antibody is not suitable for use in my assays. As a result, replacing the antibody's batch, form or brand was the next step.

A second antibody PKAR1 α (C-14): sc-18800 was therefore selected. It was sourced and proved to be better. The bands were darker producing a strong signal on a bright background. C-14 is a polyclonal antibody. Polyclonal antibodies recognize more epitopes on the target protein than monoclonal antibodies and we showed that this antibody gives the expected results. The bands were more constant at the expected molecular weight (Figure 3.1C and 3.1D). Protein bands were clearer and more intense than with the first PKAR1 α antibody (3546C2a). They were easily identified and distinguishable from the background. All blots gave the same result. Therefore, this antibody is selective and reproducible.

PKAR1 α protein is expressed in human platelets and is altered by platelet activation (Figure 3.1D). Although the expected molecular weight of PKAR1 α is 48kDa, we consistently observed doublet at 48kDa and 55kDa under reducing conditions. The bands observed in resting or activated samples of platelet lysate are different in intensity. Using the UVP imaging system, the maximum intensity of each band was measured (Figure 3.2).

The intensity of the upper band (55kDa) in TRAP-activated platelet lysate (corresponding to the sample labelled as S2 in Figure 3.1) is the highest followed by Pal-FF activated platelet lysate (S3) and then resting platelet lysate (S1). This was seen in 5 independent experiments (Figure 3.2). Moreover, western blots showed that bands' intensity is greatest upon treatment with TRAP and Pal-FF peptide. This was found also in PKAR1 α lower band (48kDa); TRAP-activated (S2) has the greatest intensity followed by Pal-FF activated (S3) and resting platelets (S1).

The band intensity of the 55kDa is 4.54% of the total intensity percentage (100) of the 48kDa band in the resting platelet lysates. In contrast, following platelet activation by TRAP, the intensity increased to 11.15% of the intensity of the total PKAR1 α protein in this sample, suggesting that an activation-dependent modification occurred which resulted in increasing the molecular weight of PKAR1 α from 48kDa to 55kDa.

Thus, we have shown that PKAR1 α exists in platelets in one or two isoforms with a molecular weight of 48 or 55 kDa and varies in intensity following platelet activation with TRAP. The antibody (3546C2a) was of insufficient quality to allow detection of this protein in platelet lysates, but a second antibody (C-14) proved to be a better choice.

PKAR1 α protein is a thiol-dependent tetrameric complex. The predicted molecular weight of the PKA tetramer that is comprised of two regulatory and two catalytic subunits (Bossis and Stratakis, 2004) is 160kDa (The Universal Protein Resource). Dithiothreitol (DTT) is a thiol-reducing reagent. Most of our experiments use DTT-treatment prior to SDS-PAGE sample separation to allow detection of the specific PKAR1 α subunit.

In some experiments, we analysed non-reduced samples. Results in these experiments showed that when not adding the reducing reagent DTT to the samples, bonds remain intact and bands were observed at high molecular weight 160kDa, as seen in Figure 3.1C. This confirms that the protein being identified in western blots is indeed the PKAR1 α protein as it is located at 48/55kDa when gels run under reducing conditions but is located at 160kDa when gels were run under non-reducing conditions. 48/55kDa is consistent with the expected molecular weight for the PKAR1 α monomer, whereas 160kDa is consistent with the identification of a tetramer.

In conclusion, PKAR1 α presents as doublet in western blots of human platelets (n=5). The difference in the relative band intensities for the 48kDa and the 55kDa components show activation induced changes. Moreover, choosing the suitable antibody, gel percentage and working techniques produce a clean western blot.

3.2.2 PINCH-1- Identification of PINCH-1 (LIMS1) in human platelets

The antibody (D-18: sc-47912) was used to probe for PINCH-1 in human platelet lysates, (PINCH-1, SCBT). This antibody is new in research as there are no previous publications using it. Therefore, we looked at the supplied data sheet from the company and found it to have good reactive components. The band illustrated was dense with a good intensity on a bright background, thus we acquired the antibody.

PINCH-1 reactive bands were found in washed platelet lysates. Western blot experiments showed PINCH-1 protein a single bands at a molecular weight of approximately 37kDa (n=6) as seen in Figure 3.3. In order to improve protein detection we had to increase protein loading

concentration up to 20 μg per lane. The bands looked defined on a bright background. We consistently got bands with different intensities: The resting platelet lysate band was the most intense band, then the Pal-FF activated sample and the least intense was the TRAP-activated sample. This was observed in 6 separate experiments as demonstrated by the densitometry data in Figure 3.3C. The reason behind low band intensity is uncertain. In order to investigate if decreased band intensities in activated platelet lysates was due to technical difficulties with lysate preparation, western blots of integrin α_{IIb} were routinely analysed in parallel (Figure 3.5A and 3.5B). Bands intensity for both PINCH-1 and α_{IIb} were measured using ImageJ method programme. In all cases, the band intensities for α_{IIb} was identical across all lysates (Figure 3.5B). This confirms that the decrease in PINCH-1 band intensity observed in activated platelets is not due to difficulties in solubilizing lysates from activated platelets but rather is due to modification or degradation of PINCH-1 protein.

To conclude, PINCH-1 protein is expressed in human platelets. PINCH-1 (D-18) antibody is an appropriate antibody for use in these studies and gives reproducible results. Adjusting the protein loading concentration is important for better band detection. Finally, the observed intensity of platelet PINCH-1 protein is altered with the activation states of the platelets. Thus, PINCH-1 appears to be degraded in response to cellular activation. This strongly suggests that this protein plays an active role in platelet activation. The fact that the integrin-derived peptide Pal-KVGFFKR also affects PINCH-1 protein intensity is strongly suggestive for a potential role for this protein in integrin activation. Moreover, integrin must somehow stabilize the activation dependent proteolytic degradation of PINCH-1. Again, this is supportive of a role for PINCH-1, either directly or indirectly, in the control of integrin activation.

3.2.3 Hic-5- Identification of Hic-5 (TGFB1I1) in three different platelets' statuses.

Western blot experiments were done to detect the presence of the protein Hic-5 in human platelets. Two different clones were used, (F6: sc-137051) (Hic-5, SCBT-b) and (H75: sc-28748) (Hic-5, SCBT-a).

The first Hic-5 antibody (F6) was not an ideal choice for Hic-5 protein detection. The initial experiments displayed faint bands. After four unsuccessful attempts (Figure 3.4A) with no consistent results, we concluded that the Hic-5(F6) antibody is not selective, reproducible or specific and was not used in any further experiments.

Therefore, the second antibody Hic-5(H75) was used. Hic-5(H75) antibody was used in a previous study in platelets (Rathore et al., 2007). Western blot experiments were successful in detecting Hic-5 protein bands. Hic-5 protein is present in human platelets and the reactive bands are persistently found as a doublet band with molecular weight of 45kDa and 55kDa (Figure 3.4B). This observation is consistent with previous studies (Osada et al., 2001) and others, where it was hypothesised that Hic-5 protein consists of more than one isoforms.

It was observed too that Hic-5 bands differ in intensity depending on the activation status of the platelet. The 55kDa band of the resting platelet sample for Hic-5 protein was observed to be weaker than the activated bands at 55kDa (TRAP and Pal-FF samples). Furthermore, the activated samples (TRAP-activated and Pal-FF) have equal intensities. On the other hand, the intensity of the 45kDa bands is highest in resting platelets and decreases in intensity following activation with TRAP and Pal-FF (Figure 3.4C).

In summary, Hic-5 protein exists as two isoforms in resting and activated platelets. The relative intensity of the Hic-5 protein bands is altered in activated platelets. Also, Hic-5(H75) antibody is selective, reproducible and specific.

3.2.4 α_{IIb} (SZ.22) - The model protein

SZ.22: sc-59923 antibody was used to probe for the α_{IIb} -chain of the integrin $\alpha_{IIb}\beta_3$ in human platelets (aIIb, SCBT). Anti- α_{IIb} detects the protein at 136kDa. We could not locate previous publications about sc-59923 (SZ.22) antibody in particular, but there were other antibodies that corresponds to the integrin α_{IIb} from different companies.

One of the project's aims is to detect the co-localisation of the proteins PKAR1 α , PINCH-1 and Hic-5 in resting and activated platelets with the integrin $\alpha_{IIb}\beta_3$. The integrin $\alpha_{IIb}\beta_3$ exists abundantly in platelet cells. It is present on the plasma membrane of circulating platelets in an inactive or quiescent state. The α_{IIb} chain of it has the distinctive aminoacid sequences KVGFFKR. Therefore, we used it as a control antibody to compare our results with.

SZ.22 antibody was used in western blot experiments. The integrin α_{IIb} chain was detected in human platelets. SZ.22 antibody is selective and reproducible and is reliable in experiments. It possesses precise standard results. The bands are consistently between 120kDa and 140kDa, clear, thick, and always appear as a strong signal on a bright background (Figure 3.5).

The experimental conditions used for SZ.22 antibody are the same for every adjacent antibody that was used in parallel, such as time for primary and secondary antibody incubation, blocking conditions and gel percentage.

To conclude, the steps that were used during western blots experiments were to identify if the antibody is in any previous publications and if not, to examine using the data sheet with trial-by-error approach. Next, we wanted to establish the appropriate gel percentage to improve the working techniques. In addition, the proteins PKAR1 α , PINCH-1 and Hic-5 were detected in resting and activated platelets. SZ.22 is an ideal antibody that can be used in platelet studies as a positive loading control.

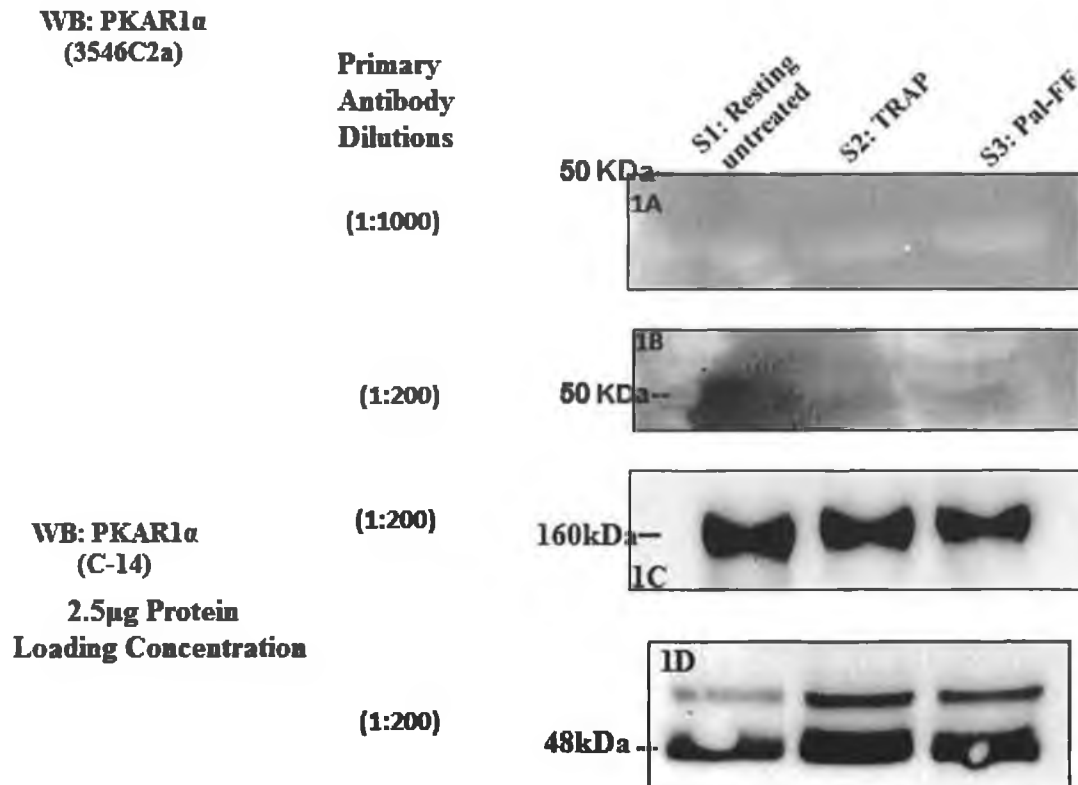


Figure 3.1. PKAR1 α protein is expressed in human platelets at 48kDa as doublets.

Washed platelets were left untreated or were treated with either TRAP (10 μ M) or Pal-FF peptide (100 μ M) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and resolved in SDS-PAGE under reduced conditions and probed for PKAR1 α as follows: (A) 1:1000 dilution of PKAR1 α (3546C2a) antibody or (B) 1:200 dilution of PKAR1 α (3546C2a) antibody to detect PKAR1 α protein. In panel (C) in non-reduced platelet lysate a band is observed at 160kDa using PKAR1 α (C-14) antibody at (1:1000) dilution. The same samples are analyzed under reducing conditions in panel (D) using a 1:200 dilution of PKAR1 α (C-14) antibody. In all samples 2.5 μ g of protein was loaded per lane. The western blot shown is representative of one of five independent experiments.

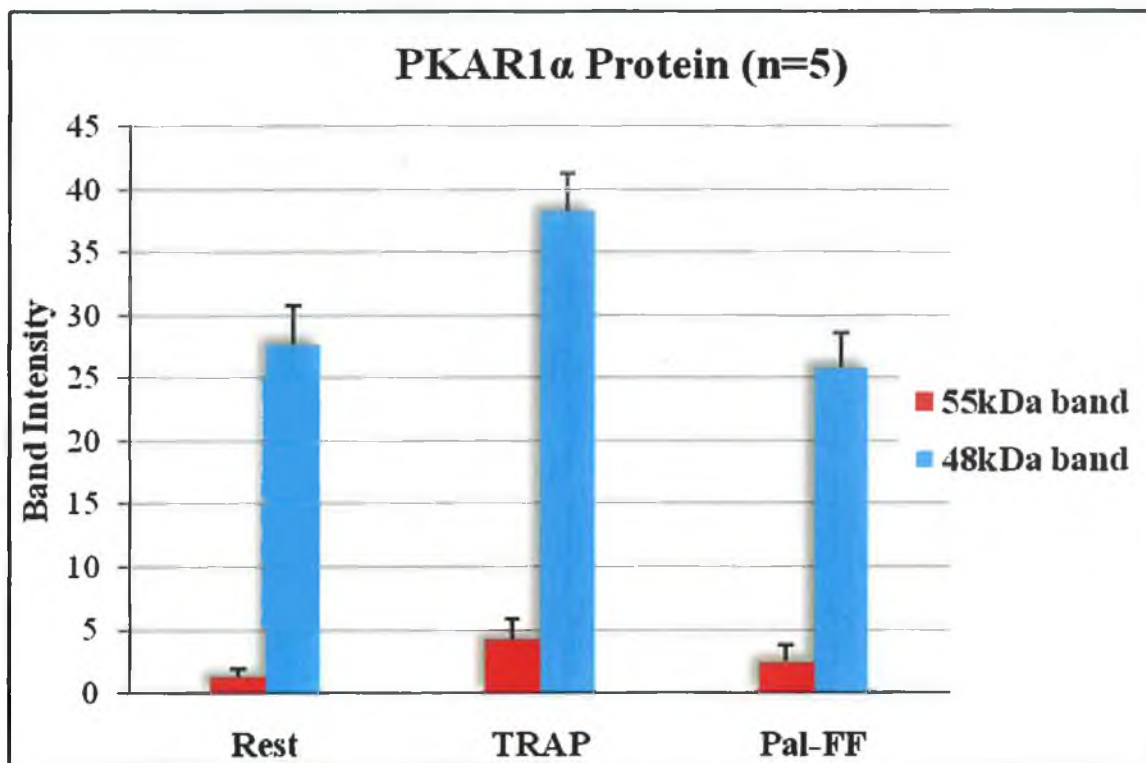
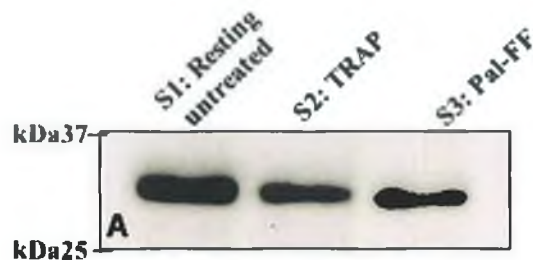


Figure 3.2. The difference in bands intensity (48 and 55 kDa) for PKAR1 α following platelet activation. Band's intensity of PKAR1 α protein identified by the C-14 antibody at 48 kDa (blue bars) and 55 kDa (red bars) was measured in inactive platelets (resting) and active platelets (TRAP and Pal-FF). Data are represented as mean \pm SEM for 5 independent experiments.

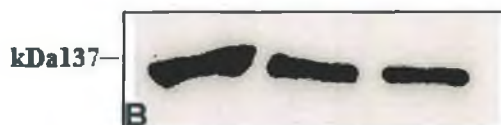
WB: PINCH-1

2.5µg Protein
Loading Concentration



(1:200) Primary
Antibody Dilution

WB: SZ.22



(1:1000) Primary
Antibody Dilution

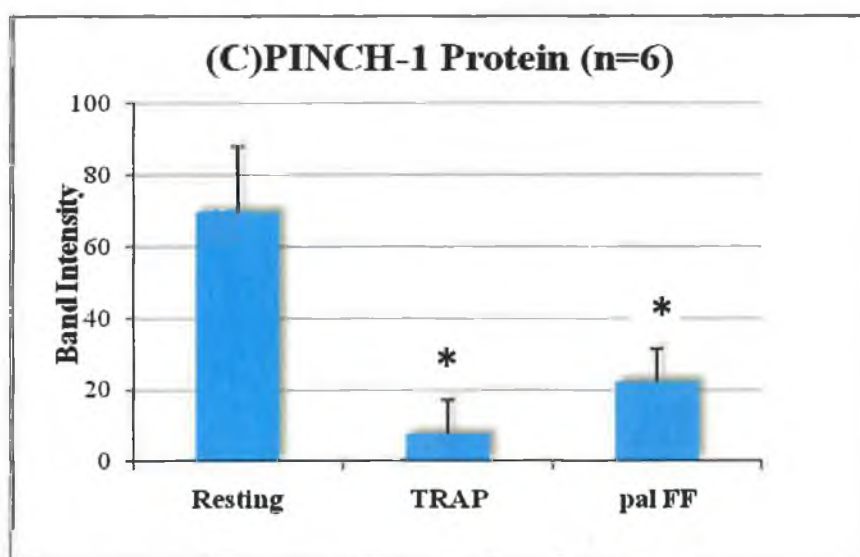


Figure 3.3. PINCH-1 protein is expressed in human platelets at 35kDa.

Washed platelets were left untreated (resting) or were treated with either TRAP (10µM) or Pal-FF peptide (100µM) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and resolved in SDS-PAGE under reduced conditions. (A) PINCH-1 protein was detected using western blot analysis. (B) α_{IIb} protein was detected in parallel for every protein. In all samples 2.5ug of protein was loaded per lane. The western blot shown is representative of one of three independent experiments. (C) The difference in bands intensity of PINCH-1 protein following platelet activation. The intensity of PINCH-1 protein bands was measured in inactive platelets (resting) and active platelets (TRAP and Pal-FF). Data are represented as mean \pm SEM for 6 independent experiments. * indicates a significant difference of $P < 0.05$ compared to resting platelets, as determined by two-tailed student T-test

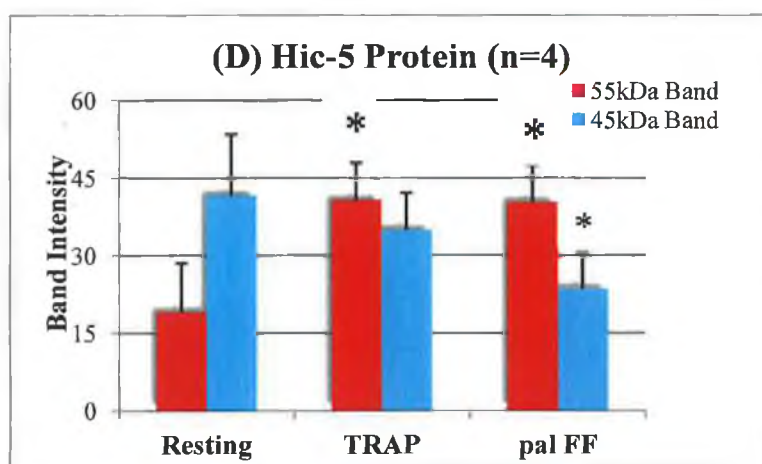
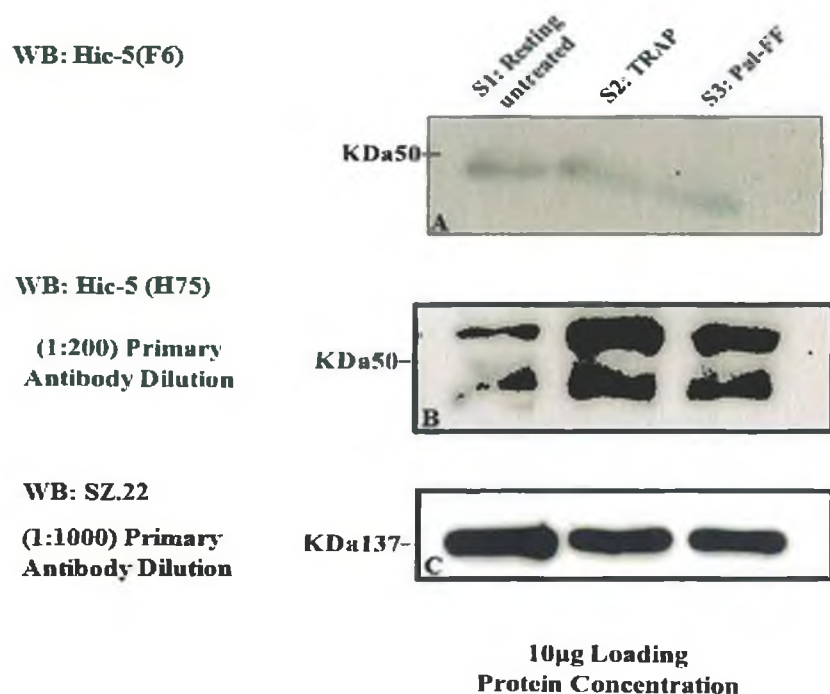


Figure 3.4. Hic-5 protein is expressed in human platelets at 55kDa.

Washed platelets were left untreated (resting) or were treated with either TRAP (10µM) or Pal-FF peptide (100µM) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and resolved in SDS-PAGE under reduced conditions. Hic-3 protein was detected in western blot analysis using (A) Hic-5(F6) antibody at (1:200) dilution and (B) Hic-5(H75) antibody at (1:200) dilution. (C) α_{IIb} protein was detected in parallel for every protein. (B-C) In all samples 10ug of protein was loaded per lane. The western blot shown is representative of 4 independent experiments. (D) Bands' intensity of Hic-5 protein after platelet activation using Hic-5(H75) antibody. Band's intensity of Hic-5 protein at 45kDa (blue bars) and 55kDa (red bars) was measured using Image J software in inactive platelets (resting) and active platelets (TRAP and Pal-FF). Data are represented as mean \pm SEM for 4 independent experiments. * indicates a significant difference of $P < 0.05$ compared to resting platelets, as determined by two-tailed student T-test

WB: SZ.22(α IIb)

(1:1000) dilution

2.5 μ g protein
concentration

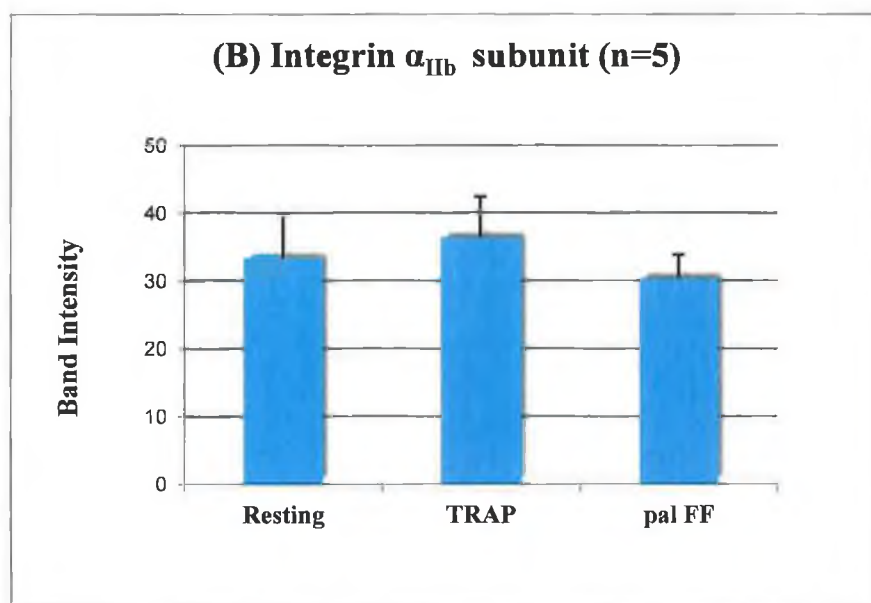


Figure 3.5. Human Platelets express the integrin α IIb β ₃ at 137kDa.

(A.) Washed platelets were left untreated (resting) or were treated with either TRAP (10 μ M) or Pal-FF peptide (100 μ M) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and resolved in SDS-PAGE under reduced conditions. The α IIb protein was detected using SZ.22 (α IIb) antibody in western blot analysis. In all samples 2.5 μ g of protein was loaded per lane. The western blot shown is representative of 4 independent experiments. (B) Bands' intensity of α IIb protein after platelet activation. Band's intensity of α IIb protein was measured in inactive platelets (resting) and active platelets (TRAP and Pal-FF). Data are represented as mean \pm SEM for 5 independent experiments.

m	Hyperlink to raw data	Gene name	Protein name	n	Rest	Act	Lys
a	ENSP00000290378	ACTC1	Actin, α -cardiac muscle 1 [a]	4	11 917 (48)	30 856 (56) ^a	27 47 (8)
a	ENSP00000190400	ACTN1	α -Actinin 1 [a]	5	38 620 (168) ^a	12 597 (147) ^a	51 08 (52)
a	ENSP00000356537	ACTN2	α -Actinin-2 [a]	3	1286 (34) ^a	14 (2)	0 (0)
a	ENSP00000252889	ACTN4	α -Actinin-4 [a]	5	17 688 (75) ^a	5858 (67) ^a	20 4 (24)
a	ENSP00000348142	ATP4C1	ATP synthase subunit c [a]	4	1299 (54) ^a	925 (17)	13 6 (2)
a	ENSP00000280012	BMX2	Bridging integrator 2	5	350 (20) ^a	178 (3) ^a	88 (2)
a	ENSP00000280326	CCTB	T-complex protein 1 subunit epsilon [a]	5	372 (10) ^a	245 (11) ^a	13 5 (2)
a/P	ENSP00000333873	CIB1	Calmodulin and integrin-binding protein 1 [a, b, c]	3	405 (12) ^a	0 (0)	0 (0)
a	ENSP00000315120	CLU	Clusterin precursor	4	388 (14) ^a	323 (5) ^a	0 (0)
a	ENSP0000031552	CTSA	Cathepsin A [a, b]	4	113 (4) ^a	186 (6) ^a	0 (0)
a	ENSP00000348785	DCTN2	Dyractin subunit 2	4	188 (6) ^a	847 (5)	0 (0)
a	ENSP00000246089	DSTN	Destrin	4	124 (9) ^a	93 (4)	0 (0)
a	ENSP00000226957	EEF1B2	Elongation factor 1 β [a]	4	139 (6) ^a	195 (4) ^a	0 (0)
a	ENSP00000282354	EHF1	EH domain containing protein 1 [a, b]	3	116 (2) ^a	254 (5) ^a	0 (0)
a	ENSP00000318772	EHF2	EH domain containing protein 2 [a, b, c]	5	435 (11)	439 (19) ^a	33 2 (6)
a	ENSP00000353467	FLNA	Filamin A	5	49 880 (287) ^a	29 864 (220) ^a	14 880 (211)
a	ENSP00000348119	FLNB	Filamin B [a, b]	4	180 (11) ^a	191 (2)	0 (0)
a	ENSP00000327146	FLNC	Filamin C [a]	3	1048 (15) ^a	548 (5)	58 7 (3)
a	ENSP00000360872	GNB1	G-protein GNB1 (SYG1) subunit β 1 [a, b]	3	628 (6) ^a	115 (4) ^a	0 (0)
a	ENSP00000373481	GPTX1	Glutathione peroxidase 1 [a, b]	4	412 (23) ^a	91 (3) ^a	0 (0)
a	ENSP00000248563	HSPB1	Heat shock 70 kDa protein	5	317 (13) ^a	1237 (13) ^a	18 2 (5)
a	ENSP00000340019	HSPD1	Heat shock protein 80 kDa	4	388 (10) ^a	267 (19) ^a	85 (1)
a	ENSP00000229319	LDAHA	L-lactate dehydrogenase A/C	5	568 (22) ^a	889 (34) ^a	41 5 (8)
a/P	ENSP000003001775	LMNB1	PMCH protein	5	917 (26) ^a	654 (16) ^a	29 4 (10)
a	ENSP00000218181	MYH9	Myosin heavy chain 9	5	5086 (141) ^a	2538 (44) ^a	18 16 (35)
a/c	ENSP00000327437	MYL8B	Myosin light chain 8B	3	1454 (35) ^a	44 (3)	27 (1)
a	ENSP000003081540	MYL6	Myosin light polypeptide 6	4	8598 (17) ^a	6250 (18)	19 04 (8)
a	ENSP00000279022	MYL9	Myosin regulatory light polypeptide 9	4	4047 (66)	235 (38) ^a	890 (14)
a	ENSP00000300556	PCBP1	Poly (IC) binding protein 1	5	395 (18) ^a	485 (10) ^a	14 4 (2)
a/P	ENSP00000335084	PPP1CB	Serine/threonine protein phosphatase 1 [a, b]	3	55 (2)	233 (5) ^a	14 4 (2)
a/P	ENSP00000221128	PPP2CB	Serine/threonine protein phosphatase 2A [a, b]	3	84 (3) ^a	212 (6) ^a	0 (0)
a	ENSP00000351410	PRKAR1A	cAMP dependent protein kinase type 1 α regulatory subunit [a, b]	3	134 (2)	345 (4) ^a	0 (0)
a/c/P	ENSP000003005908	RNF181	RING finger protein 181 [a, b]	3	60 (4) ^a	0 (0)	0 (0)
a	ENSP00000346402	RNMT	Ribonuclease inhibitor [a, b]	4	158 (7) ^a	102 (1)	0 (0)
a	ENSP00000221413	RUVBL2	RuvB-like 2 [a, b]	2	0 (0)	225 (4) ^a	0 (0)
a/P	ENSP00000221830	TGFB1	TGF β -1 inhibitory-associated protein 1 [a]	3	368 (8) ^a	0 (0)	0 (0)
a/P	ENSP00000355117	TGFB11	TGF β -1 induced transcript 1 protein [a, b]	3	223 (2) ^a	102 (5) ^a	0 (0)
a/P	ENSP00000316029	TUB1	TUB1	5	28 804 (322) ^a	3975 (277)	70 92 (188)
a	ENSP00000336799	TUBAL3	Tubulin α ubiquitous [a]	5	5251 (37)	11 000 (15) ^a	77 85 (12)
a/c	ENSP00000259925	TUBB	Tubulin β chain [a]	5	6469 (41) ^a	11 808 (84) ^a	31 50 (17)
a	ENSP00000284273	UBASH3B	STS 1 ubiquitinase and SH2 domain protein B	5	153 (8) ^a	198 (12)	92 (3)
a	ENSP00000229288	USP5	Ubiquitin carboxyl-terminal hydrolase 5 [a, b]	4	48 (6) ^a	0 (0)	0 (0)
a	ENSP00000246932	VASP	Vasodilator-stimulated phosphoprotein [a, b]	4	38 (2)	414 (16) ^a	12 7 (3)
a	ENSP00000371890	WDR1	WD repeat-containing protein 1	5	2021 (30)	2356 (44) ^a	15 41 (10)

Proteins associated with the ubiquitin pathway are presented in bold. The methods (m) returning a positive protein identification are abbreviated as follows: a, peptide affinity chromatography; c, protein chip; P, PPI databases. Proteins identified by more than one approach are indicated in bold. Letters in brackets after the protein name hyperlink to spectral information for discriminatory peptides identifying the respective protein. n gives the total number of experiments (out of five) in which a protein is identified. The three right-most columns give the mean intensities (and the total unique spectra in parentheses) for a protein identified in retained eluting (rest) and activated (act) lysate samples as well as in lysates (lys) prior to chromatographic separation. The results of paired t-tests comparing relative mean intensities of a protein in a retained subpopulation with those in the lysate prior to peptide affinity chromatography are indicated by $p < 0.05$ or $p < 0.1$.

Table 3.1. List of 44 proteins that were identified by LC-MS/MS. The table shows the mean intensities (and total peptide counts per protein, shown in parentheses) for proteins that were found in resting and activated platelet samples and in platelet lysates. Highlighted in bold text (and coded in the leftmost column) are proteins that have been previously identified in the literature (P) or which were identified in protein chip experiments (c). Proteins coded as (a) were identified in peptide affinity chromatography experiments (Raab et al., 2010).

3.3 Discussion

In this chapter we have identified antibodies adequate for detecting the proteins PKAR1 α , PINCH-1 and Hic-5 in human platelets. Western blot analysis was used to demonstrate the presence of these proteins in lysates from inactivated (resting) human platelets, TRAP-activated and Pal-FF activated platelets. The most abundant platelet-protein, integrin α IIb, was analysed in parallel with the three novel proteins. Furthermore, proteins' changes were recognized upon cellular activation and studied using densitometry analysing softwares. Treatment with a strong platelet agonist such as TRAP (10 μ M) or a weak agonist such as Pal-FF peptide (100 μ M) for three minutes alter protein expression and distribution.

3.3.1 PKAR1 α - Identification of PKAR1 α in human platelets

The difference in the antibody type can influence to an extent the results produced in western blot analysis. That was illustrated in two PKAR1 α antibodies that have been used. The first (3546C2a) is monoclonal (Figures 3.1A and 3.1B) and the second (C-14) is polyclonal antibody (Figures 3.1C and 3.1D). Polyclonal antibodies often have the advantage of better antigen recognition as they represent a group of epitopes that are available to bind antigens. Thus, polyclonal antibodies result in a higher chance of immunogen detection as they recognize multiple epitopes on a single protein (Bordeaux et al., 2010). In contrast, monoclonal antibodies will only recognize a single epitope. Moreover, if the single epitope recognized by the monoclonal antibody is complex, it may be denatured by the SDS-PAGE preparation procedure. However, commercial suppliers usually declare in their data sheets if antibodies are suitable for western blots.

It was detected that the isozymes (α and β) of regulatory subunit I of protein Kinase A exists in human neoplastic B cell line (Reh) (Tasken et al., 1993). Moreover the regulatory subunit I α is distributed almost throughout the whole body (Bossis and Stratakis, 2004). In a previous study by our group using peptide-affinity chromatography and LC-MS/MS analysis, it was identified that PKAR1 α was present in human platelets, demonstrated in Table 3.1 (Raab et al., 2010). Although this study showed it to be present in low abundance, it was considered to be interesting as its binding to the integrin-derived peptide differed according to the activation state of the platelet lysate. By doing Western blot analysis this study further proves that PKAR1 α protein exists in human platelets.

In the experiments, there were two samples subjected to reduced and non reduced conditions, with and without DTT respectively. Results in our experiments showed that in samples without the reducing agent DTT the PKA complex remains intact and bands were identified at the high molecular weight of 160kDa (The Universal Protein Resource) consistent with its known tetrameric structure, as seen in Figure 3.1C. In contrast, PKAR1 was identified at the expected molecular weight range of 48kDa when samples were reduced with DTT to monomers. This confirms that the proteins identified in my studies is indeed the PKAR1 α monomer at 48kDa in its reduced condition and the PKA complex at 160kDa in its non-reduced form. Therefore, PKAR1 α is a thiol-dependent complex and is recognized in platelet lysates by the (C-14) antibody.

The bands of PKAR1 α appear as a doublet at 48kDa and 55kDa. This was consistently observed in western blot analysis (n=5) as seen in Figure 3.1D. The reason behind the doublet remains unknown. The existence of genetically distinct isoforms is not documented. Therefore, proteins of separate discrete molecular weights could result from enzymatic modification such as phosphorylation, post translational modification or protease action (enzyme cleavage). For example, the higher band at 55kDa could be a product of ubiquitination, as the difference in molecular weight between the doublets is 7-8 kDa, and ubiquitination results in the addition of an 8kDa ubiquitin moiety to a protein. Also, PKAR1 α has been associated with the ubiquitin pathway by other groups (Lignitto et al., 2011).

The intensity of the lower bands at 48kDa was always the same for the three samples. It was intense in all blots, but the upper bands at 55kDa were not as intense as the lower bands in all cases. The band intensity from the TRAP-activated sample was the strongest for both bands (Figure 3.1D).

The 48kDa band that is the main expected molecular weight of the protein PKAR1 α is intense in all blots. The intensity of the 48 kDa changes from resting to activated platelets. In contrast, the 55 kDa was not intense as the lower band (48kDa). The intensity of 48kDa is greatest in the TRAP-activated sample followed by the resting then the Pal-FF activated samples. On the other hand, the Pal-FF activated sample is greater than the resting sample in intensity for the 55 kDa band (Figure 3.2). The intensity's results were determined using UVP imaging system (Vision WORKsLS).

To summarize, PKAR1 α protein is expressed in human platelets, as a doublet at 48kDa and 55kDa. The expression pattern is altered upon cellular activation. PKAR1 α (C-14) antibody is adequate for western blot analysis.

3.3.2 PINCH-1- Identification of PINCH-1 (LIMS1) in human platelets

Western blot analysis was done to probe PINCH-1 protein from human platelet lysates ($n \geq 6$). Experiments showed that PINCH-1 protein is present in human platelets. These results were consistent with the data obtained in the Mass spec experiments published by our platelet function group (Raab et al., 2010). PINCH-1 protein appeared as single band at approximately 37kDa.

We found in the study that the intensity of the bands' was different from resting to activated platelet lysate samples; the band intensity was the highest in resting platelets followed by the activated samples (Pal-FF then TRAP).

The decrease in band intensity is most likely associated with protein degradation. Results showed that the bands degrade upon platelet activation. TRAP is referred to as a strong activator of platelets and PAL-FF is regarded as a weak activator of platelets. Consistent with this, the degree of degradation of PINCH-1 protein is higher with TRAP than with PAL-FF (Figure 3.3.C) suggesting that the association of PINCH-1 with the integrin via the KVGFFKR region may play a role in PINCH-1 degradation.

Western blot results of PINCH-1 showed that the bands degraded after platelet activation. To exclude that the protein possibly degraded due to experimental artefacts such as inadequate protein solubilization; all the three samples were treated under the same conditions (reduced and vortexed to ensure adequate protein solubilising). A control western blot was analysed in parallel with the anti- α_{IIB} antibody (SZ.22). This was to demonstrate almost equal levels of protein α_{IIB} was present in the lysates (Figure 3.3B and 3.5B). Moreover, previous western blot experiments of PKAR1 α and Hic-5 showed more intense bands upon platelet activation. Suggesting more likely that the changes in PINCH-1 band intensity following platelet activation is caused by protein degradation.

In summary, the observed intensity of platelet protein PINCH-1 is altered with the activation status of the platelet. This strongly suggests that this protein plays an active role in platelet

activation. The fact that the integrin-derived peptide; Pal-KVGFFKR also affects PINCH-1 protein intensity is suggestive for a potential role of this protein in integrin activation also.

3.3.3 Hic-5- Identification of Hic-5 (TGFB1I1) in three different platelets' statuses.

Hic-5 protein is present in the resting and activated states of the human platelets. Western blot analysis of n=4 of human platelet lysates using anti-Hic-5 polyclonal antibody showed the presence of two bands (or doublet) at approximately 45 and 55KDa. Both bands are well-defined band and not vague. This supports other studies that mentioned that Hic-5 may exist as multiple isomers (Hagmann et al., 1998); (Osada et al., 2001). Activation of platelets with TRAP and Pal-FF peptide causes a shift in the relative intensities of the two Hic-5 reactive bands (Figure 3.4B). The intensity of the lower band (45kDa) decreases with activation whereas activation causes the opposite for the the intensity of the upper band (55kDa); it increases. This is consistant with a substandial activation-induced post-translational modification resulting in a shift in molecular weight. As for PKAR1 α protein, the magnitude of the shift in molecular weigh is consistant with a potential ubiquitination reaction. However, we did not specifically investigate the possibility of ubiquitination, so can only speculate that this is the cause of the altered molecular weight.

Hic-5 is a cytoskeletal protein that moves from cytosol and membrane skeleton in resting platelets into the cytoskeleton upon $\alpha_{IIb}\beta_3$ activation (Osada et al., 2001). Researchers like Rathore and colleagues found that Hic-5 protein is phosphorylated following platelet activation and spreading on fibrinogen. However, the Hic-5 doublet in our studies are not entirely consistant with phosphorylation. Firstly, the molecular weight difference is too high (more than 5 kDa) as seen in Figure 3.4B, secondly, the doublet exist in both resting (S1) and activated (S2 and S3) platelet samples. Nevertheless, the higher molecular weight component (55kDa) has a greater intensity following platelet activation with either TRAP or Pal-FF peptide, where both have equal intensities, suggesting that some kind of post-translational modification is occurring following platelet activation. In summary, the intensity of the 45kDa band is highest in the resting platelet sample followed by the TRAP and then the Pal-FF samples. This is consistent with the result that the 55kDa band achieves greater intensity upon platelet activation, showing that the protein alters with the activation status of the platelet.

In conclusion, Hic-5 protein presents in human platelets as doublets (45 and 55 kDa). The two forms appear to be interconvertible during cellular activation. This strongly suggests that this protein plays an active role in platelet activation. In addition, Hic-5 (H75) antibody is a good choice for western blot experiments.

3.3.4 The control antibody SZ.22 (α_{IIb} subunit)

SZ.22 monoclonal antibody was used to probe the α_{IIb} subunit of the integrin $\alpha_{IIb}\beta_3$. This integrin presents abundantly in human platelets. Western blot analysis of α_{IIb} protein show consistent results of strong bands suggesting that the platelets are rich of it (Figure 3.5A). Further more, it was observed that there are similar levels of α_{IIb} protein in all different platelet statuses; resting, TRAP-activated and Pal-FF activated showing constancy and uniformity (Figure 3.5B). We concluded that the protein α_{IIb} is a reliable protein to be used as a good reference-standard in western blot analysis that is conducted on human platelet lysate samples, to allow determining the samples loaded onto gels.

In conclusion, the proteins PKAR1 α , PINCH-1 and Hic-5 are present in resting and activated platelets. PKAR1 α and Hic-5 proteins appear as doublets. The relative intensity of the protein bands alters with platelet activation, supporting a hypothesis that these proteins play a role in platelet activation with TRAP and with the integrin-derived peptide KVGFFKR. The higher molecular weight of the doublet possibly represents a post-translationally modified variant possibly due to enzymatic activity such as phosphorylation or ubiquitination. In addition, PINCH-1 protein, which is apparent as a monomer of the expected molecular weight (37kDa), is also modified by cellular activation. However, in the case of PINCH-1, the protein is apparently degraded during platelet activation. Finally, SZ.22 is an antibody that recognizes the abundant platelet protein α_{IIb} and can be used successfully in platelet studies as a positive loading control. The intensity of SZ.22 staining in western blots remains constant across all samples demonstrating that our protocol for solubilising platelet proteins is effective in yielding equivalent amounts of protein from resting and activated platelets. Moreover, this result allows us to conclude that the specific activation dependent changes we observed in PKAR1 α , Hic-5 and PINCH-1 are not artefactual but represent real changes in protein abundance, protein modification or protein degradation that occurs in response to platelet activation with TRAP or the integrin-derived peptide KVGFFKR. Thus we have demonstrated that relative to α_{IIb} protein levels in platelets, PKAR1 α levels appear to increase

following platelet activation whereas PINCH-1 levels appear to decrease. It is likely that the changes in PINCH-1 band intensity following platelet activation is caused by protein degradation. It is more difficult to explain the apparent increase in intensity of PKAR1 α . It is possible that the antibody chosen recognizes an epitope that is better exposed following some activation-induced post translational modification.

Chapter 4

Protein Interaction with the Integrin $\alpha_{\text{IIb}}\beta_3$ in Platelets

4.1 Introduction

In a proteomic study done by the platelet function group here in RCSI, it was found that there are numerous possible platelet integrin $\alpha_{IIb}\beta_3$ binding proteins with unidentified roles within the platelet cytoplasm (Raab et al., 2010). Three proteins in particular, identified by their affinity interaction with the integrin regulatory motif, KVGFFKR by LC-MS/MS were PKAR1 α , PINCH-1 and Hic-5. These cytoplasmic proteins were identified as interesting proteins and were worth pursuing as potential integrin regulatory proteins. These proteins interact with the conserved motif KVGFFKR of the integrin α_{IIb} subunit according to our mass spectrometry data but may do so as primary, secondary or tertiary interactors of the integrin α_{IIb} subunit.

A group in the United States had previously published that Hic-5 protein does not bind to the integrin $\alpha_{IIb}\beta_3$ (Liu et al., 1999). In their study, they focused on paxillin binding to other alpha integrins but examined Hic-5 binding to the platelet integrin as a small part of their study. However, we were confident that for Hic-5, our mass spec data was strongly suggestive of an interaction of this protein with the integrin cytoplasmic tail. We therefore set out to test the hypothesis that PKAR1 α , PINCH-1 and Hic-5 may bind to the integrin α_{IIb} subunit by using co-immunoprecipitation as a technique. Very simply, the idea behind this application is to remove one protein (protein X) from a cell and subsequently try to detect if another protein (protein Y) is attached to this, suggesting a biological interaction.

To examine this hypothesis we did the following co-immunoprecipitation experiments:

(1) The platelet integrin $\alpha_{IIb}\beta_3$ was immunoprecipitated (IP) from washed platelet lysates with the mouse antibody (SZ.22) which is raised against the integrin α_{IIb} -chain. Following this IP, we probed for the three proteins of interest thought to be integrin interactors: (A) PKAR1 α (B) PINCH-1 and (C) Hic-5 by western blot analysis. The antibodies used in this part of the study have been characterized in Chapter 3 of this thesis. Western blot analysis of integrin α_{IIb} in the immunoprecipitated samples served as a positive control. In parallel, platelet lysates were subjected to immunoprecipitation by a control IgG antibody. This served as a negative control. Finally, samples of whole platelet lysates were separated on the SDS gels and analysed in each western blotting experiment. The identification of a protein band in these lysate samples was essential to confirm the the exact position of the protein-of-interest in the western blot, and to act as an additional positive control.

(2) In a separate series of experiments, Hic-5 protein was immunoprecipitated from platelet lysates by using a rabbit anti-Hic-5 antibody. We immunoprecipitated (IP) with Hic-5 to study the hypothesis from both angles. Then we western blotted for (A) Hic-5 (B) Integrin α_{IIb} with SZ.22 and (C) GpIb α antibodies. We probed for Hic-5 protein to ensure there was a successful pull down but also with anti-integrin antibody (SZ.22) to try and determine if this protein co-associated with Hic-5. Since it was found in an earlier study that Hic-5 protein interacts with a different platelet adhesion molecule, GPIb α (Arthur et al., 2011), we also probed for this protein in the Hic-5 immunoprecipitation samples. This provided us with an extra positive control for our experiment, helping us to verify the effectiveness of our protocol. However, due to the lack of time, we could not study the interaction between the proteins PKAR1 α , PINCH-1 and Hic-5 or immunoprecipitate with PKAR1 α and PINCH-1 antibodies.

The role of the proteins PKAR1 α , PINCH-1 and Hic-5 in human platelets is unidentified. Therefore, in this chapter we aimed only to study the interaction between the integrin α_{IIb} -subunit and the proteins PKAR1 α , PINCH-1 and Hic-5. In more detail, I will now describe the experimental conditions in preparing the platelets for a co-IP experiment.

Methods

There were nine samples in each experiment. For each experiment, three separate platelet preparations are collected and solubilised in lysis buffer. These samples are the same as those described in Chapter 3 and comprise resting platelets (R), platelets that have been activated with TRAP (T; 10 μ M for 3 minutes at 37°C) and platelets that have been activated with Pal-KVGFFKR Peptide (P; 100 μ M for 3 minutes 37°C). Each of these samples is processed as follows: (1) A small sample is taken from each lysate. Part of this is subjected to a Bio-Rad protein estimation assay and the remainder is stored in SDS Sample buffer until needed. (2) Of the remaining sample, 50% is subjected to immunoprecipitation with a non specific antibody (i.e. normal IgG) from the same species as the pull-down samples. (3) The remainder of the sample is subjected to immunoprecipitation with a specific antibody (SZ.22 or Hic-5). The protocols that were used depend on the type of the immunoprecipitating antibodies and whether it is a monoclonal or a polyclonal antibody. Protein G-Sepharose beads were used with monoclonal antibody such as SZ.22 and Protein A-Agarose beads were

used with polyclonal antibody. The following is a brief description of the Co-IP protocol as mentioned previously in Chapter 2 (Materials and Methods):

Protein A-Agarose beads were used to immunoprecipitate with polyclonal antibody and protein G-Sepharose for immunoprecipitation with the monoclonal antibody. 25 μ l for every reaction of beads was washed 3 times from slurry with ice-cold PBS and incubated rotating for 1 hour at 4°C with 0.5ml platelet (protein concentration $\sim 600 \times 10^3/\mu$ l) lysates for preclearing. The mixture was microfuged briefly to pellet the beads (72g for 30 seconds). The supernatant was divided into two and removed to fresh eppendorfs. The first eppendorf is the negative control which is incubated with an IgG-antibody and the second is for the pull-down which is incubated with the immunoprecipitation antibody. Both antibodies added for incubation of equal amounts (5 μ g/500 μ l) for Hic-5 and Rabbit IgG and (0.2-2 μ g/500 μ l) for SZ.22 and mouse IgG and kept rotating at 4°C from 2 hours to overnight. Then, equal amounts (25 μ l) of fresh washed beads were added to both eppendorfs and kept rotating for 1-2 hours at 4°C. After that, the beads were pelleted gently and washed 3 times with PBS, eluted with 2X sample buffer, boiled at 95°C for 3 minutes and spun. Samples were ready for use.

The band's intensity was determined using ImageJ software for all western blots in this chapter. The bands intensity was normalized to all samples' bands in the experiment. Thus the total intensity of the western blot bands was summed for the 9 lanes on each gel. This was assigned a value of 100. Each band was expressed as a percentage of this total. In this way, the variance between experiments was minimized.

We determined that the experiments were successful for the following reasons. First the platelet lysate samples should contain the protein of interest at the correct molecular weight. Secondly the negative control samples (IgG pull-down) should not show any bands corresponding to the proteins of interest, or the intensity of the bands should be so much less than the intensity of the bands observed in the presence of the specific antibody. Thirdly, the specific antibody should precipitate the proteins of interest. In particular, it should be able to precipitate itself. Thus when SZ.22 was used as the immunoprecipitating antibody, samples were also western-blotted with SZ.22 to ensure specific and substantial pull-down of this protein. Band intensity was determined using ImageJ software for all western blots in this chapter.

4.2 Results

4.2.1. IP with SZ.22 antibody (α_{IIb} -chain of the integrin);

We examined the binding of PKAR1 α , PINCH-1 and Hic-5 proteins to α_{IIb} -subunit, by selectively immunoprecipitating the α_{IIb} protein. Immunoprecipitating with α_{IIb} protein let us assess whether the proteins PKAR1 α , PINCH-1 and Hic-5 bind directly or not to the integrin. We successfully immunoprecipitated the α_{IIb} protein when using anti-mouse SZ.22 antibody and when we immunoprecipitated with a non-specific normal mouse IgG antibody the results were negative (as expected), demonstrating that the SZ.22 antibody is capable of specifically immunoprecipitating the integrin α_{IIb} from platelet lysates. As can be seen in Figure 4.1A, the immunoreactive band for SZ.22 runs at an identical molecular weight in total platelet lysates as in the SZ.22 pull-down experiments.

4.2.1.1 Western blot with SZ.22 antibody

In every experiment we regularly blotted with the antibody SZ.22 that probes the α_{IIb} -subunit of the integrin. SZ.22 blot is a control in the experiments. We assessed that the experiment was successful by checking there were no bands for the negative control samples and that the integrin subunit α_{IIb} in the lysates bound to the antibody SZ.22 in the pull down samples. We consistently got strong bands in the pull-down samples and no bands in the negative control samples $n=5$ (Figure 4.1A). The densitometry analysis data for α_{IIb} -subunit showed that there is a significant difference between the pull down samples and the negative control samples (Figure 4.2A). This strongly suggests that the experiments are successful. Following this success, we then wanted to determine if the integrin managed to pull-down a complex of co-associating platelet proteins by using the following antibodies: PKAR1 α , PINCH-1 and Hic-5.

4.2.1.2 Western blot with PKAR1 α antibody

Western blots of the co-IP experiment were done to detect whether PKAR1 α binds to the integrin α_{IIb} -subunit or not. Four out of six experiments were successful. Two were deemed unsuccessful because at times it would seem a lot of protein was pulled down non-specifically and the protein of interest could not be identified in the lysate samples or there was protein degradation.

The remaining results show that there is more PKAR1 α protein pulled down with the IgG antibody in the negative control samples than the pull down samples using SZ.22 antibody,

showing that PKAR1 α protein does not bind specifically to the integrin in resting, TRAP or Pal-FF activated samples as demonstrated in Figure 4.1B.

Densitometry analysis summarizes the results obtained in 4 separate experiments and illustrate that there is a lot of non-specific binding in all negative control samples (Figure 4.2B). Indeed, this protein was identified originally in a mass spec analysis of proteins binding to a KVGFFKR-peptide column. Its ability to bind non-specifically to an irrelevant antibody may be related to its ability to bind to the affinity column.

4.2.1.3 Western blot with PINCH-1 antibody

Immunoprecipitated samples were next immunoblotted with an antibody that recognizes PINCH-1 protein. Figure 4.1C shows a western blot of PINCH-1 antibody. There are almost equal amounts of protein both in the negative control samples and the pull-down samples, suggesting that, like PKAR1, there is no specific binding of PINCH-1 to the integrin α_{IIb} -chain. Densitometry analysis also showed that the band-intensity for the resting and activated samples is higher in the negative control samples than in the SZ.22 pull-downs (Figure 4.2C).

4.2.1.4 Western blot with Hic-5 antibody

Although the Hic-5 protein is identified in our proteomics experiments as binding to the integrin (α_{IIb} -subunit) regulatory peptide (Raab et al., 2010), an old study by (Liu et al., 1999) appeared to show the opposite results and states that Hic-5 does not bind to the α_{IIb} integrin. However, Hic-5 binding showed the strongest signal in our proteomics experiments, so we were keen to establish if it was able to bind to the platelet specific integrin. Therefore, we immunoprecipitated with SZ.22 antibody and probed for Hic-5 protein. Co-IP experiments (n=5) showed that the Hic-5 protein binds significantly to the α_{IIb} beads in the resting and Pal-FF-activated samples (Figure 4.1D).

In one particular experiment, there was a significant difference between the pull-down and negative control samples (Figure 4.3A). In this experiment, the resting platelet lysate sample was observed as two distinct bands at 45kDa and 55kDa representing Hic-5 protein. The bands were strong on a bright background. The corresponding band in the negative control samples was vague and faint and more consistent with the profile of the heavy chain of the antibody. There were just 2 positive control samples in this experiment; the resting and TRAP-activated platelet lysates, showing bands at approximately 55kDa. This experiment

was included in the densitometry analysis (Figure 4.2D) with the rest of the experiments. There is no representative image for the control gel SZ.22 as the gel was not well polymerized and the samples did not run smoothly (Figure 4.3B).

We conclude from these experiments (Figures 4.1D and 4.2D), that there appears to be a co-association between integrin α_{IIb} and Hic-5 in resting and Pal-FF activated platelets. The evidence for co-immunoprecipitation of Hic-5 with α_{IIb} in the TRAP-treated samples is not strong.

To summarize, there is no direct interaction between the integrin $\alpha_{IIb}\beta_3$ and the proteins PKAR1 α and PINCH-1. In contrast, the results of the SZ.22 pull-down experiments suggest that there is an interaction between the integrin $\alpha_{IIb}\beta_3$ and Hic-5 protein in resting and Pal-FF activated platelets but not in TRAP-activated platelets.

4.2.2 IP with Hic-5 antibody

In order to confirm the apparent co-association of Hic-5 with integrin α_{IIb} in resting and Pal-FF activated platelets, we repeated the co-IP experiments but changed the precipitating antibody to the anti-Hic-5 antibody (H-75: sc-28748) and western blotted against the integrin α_{IIb} . It was proven in a previous study that Hic-5 protein binds to the integrin GpIba (Arthur et al., 2011). Therefore, we used this fact in our experiments to provide us with an additional positive controls. The negative control samples were immunoprecipitated with a non specific normal rabbit IgG antibody in a similar manner to the use of mouse IgG controls for the SZ.22 immunoprecipitation experiments.

4.2.2.1 Western Blot with Hic-5 antibody

We probed every Hic-5 immunoprecipitation experiment with a mouse anti-Hic-5 antibody. It is the main control in the experiment. In these experiments we show the Hic-5 protein is immunoprecipitated with the anti Hic-5 antibody and showed, as expected, a distinct band at 55kDa corresponding to bands observed in the platelet lysate samples (Figure 4.4A). This band does not correspond to the heavy chain of the antibody because the primary antibody was from a different host species (mouse). It is noteworthy that the 55kDa band is absent or greatly reduced in samples immunoprecipitated with the negative control antibody. This demonstrates that the Hic-5 antibody is highly specific in immunoprecipitating Hic-5 protein from platelet lysates. The intensity analysis for Hic-5 protein co-immunoprecipitation showed

successful experiments, as the intensity of the pull-down bands are higher in all samples than the negative controls (Figure 4.5A).

4.2.2.2 Western Blot with GpIb α antibody

We next western blotted with a GpIb α antibody to confirm the interaction of GpIb/IX/V complex with Hic-5 protein that was first reported by (Arthur et al., 2011). This was an additional positive control in the experiment. The results showed that we successfully precipitated GpIb α (Figure 4.4B). The band's intensity confirmed that GpIb α was bound to Hic-5 proteins in both resting and TRAP-activated platelets, as there is a difference between the pull-down and negative control samples (Figure 4.5B). However, it is highly noteworthy that the integrin-derived peptide Pal-KVGFFKR inhibits this interaction.

4.2.2.3 Western Blot with SZ.22 antibody (integrin α_{IIb} -chain)

Finally, we probed the Hic-5 immunoprecipitates for the α_{IIb} protein by western blotting with the SZ.22 antibody. The results shown in Figures 4.4C and 4.5C demonstrate that there is evidence for weak binding of α_{IIb} to Hic-5. In three out of four experiments, there appears to be evidence of some co-association of α_{IIb} with Hic-5 above a background level, but the 4th experiment causes this overall result to be diminished (Figure 4.5D).

We conclude therefore that our experiments show evidence for co-association of α_{IIb} with Hic-5 in platelet lysates. This result is not the same as we obtained from co-immunoprecipitation with SZ.22 antibody (α_{IIb} -subunit) and probing for Hic-5 (Figure 4.2D) where we saw evidence for co-association of these proteins only in the resting and Pal-FF activated platelets. Further experiments will need to be performed to confirm this difference.

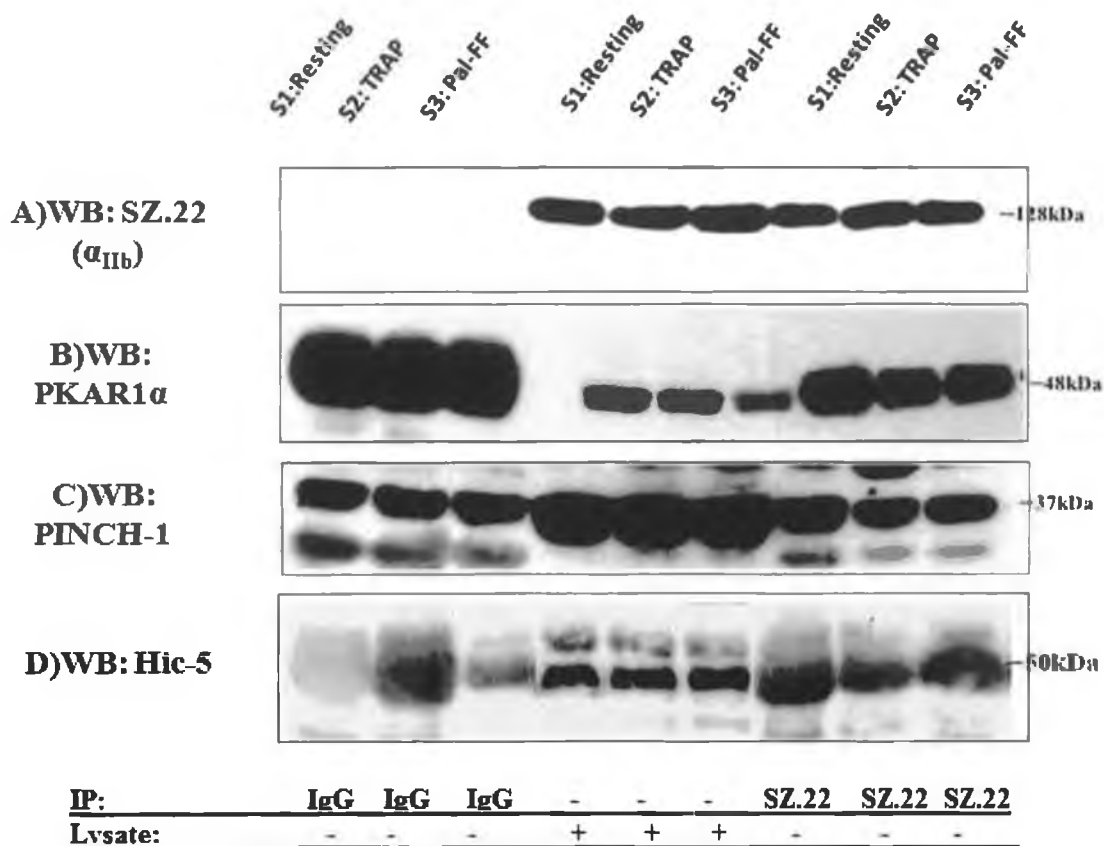


Figure 4.1. Co-immunoprecipitation of SZ.22 antibody with α_{IIb} , PKAR1 α , PINCH-1 and Hic-5 proteins. Washed platelets were left untreated (resting) or were treated with either TRAP (10 μ M) or Pal-FF peptide (100 μ M) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and immunoprecipitated with SZ.22 antibody (α_{IIb} chain of the integrin). Western blot analysis was done using (A) anti-SZ.22 antibody, (B) anti-PKAR1 α antibody, (C) anti-PINCH-1 antibody and (D) anti-Hic-5 antibody. Protein loading concentration is approximately 2.5 μ g per lane. The western blot is representative of one of 4 or more independent co-immunoprecipitation experiments. Hic-5 western blot was cropped and rearranged for presentation purpose only. An original image of the blot is attached at the end of the thesis (please see page 64).

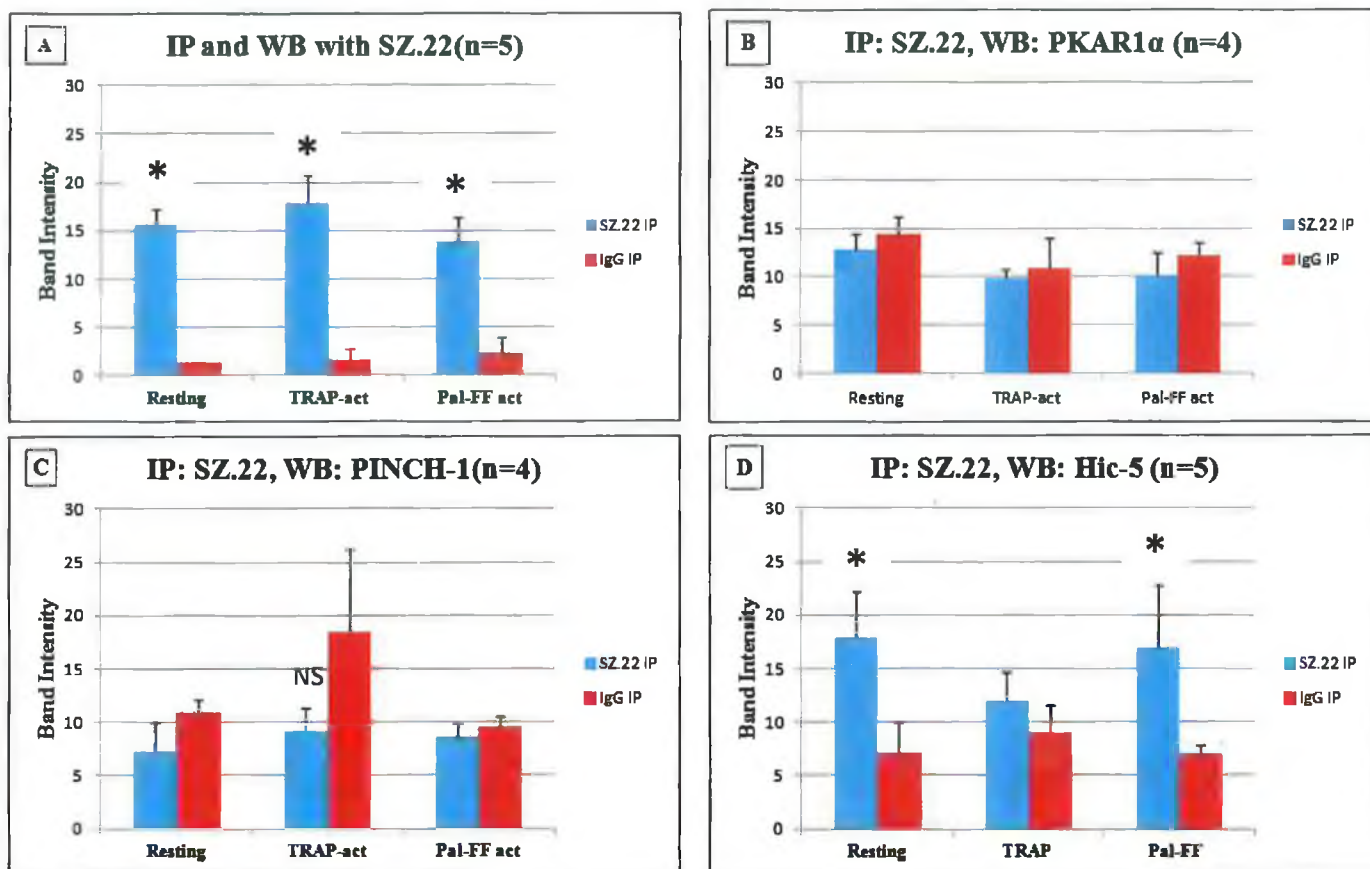


Figure 4.2. Densitometry analysis of proteins' interaction with α_{IIb} integrin.

Difference in band intensity between α_{IIb} integrin and selective proteins (A) α_{IIb} -subunit, (B) PKAR1 α , (C) PINCH-1 and (D) Hic-5 and non-specific proteins in lysates. Data are represented as mean \pm SEM for 3 independent experiments. * Indicates $P < 0.05$ compared to IgG control-IP sample, as assessed by two-tailed Student T test. NS= non significant.

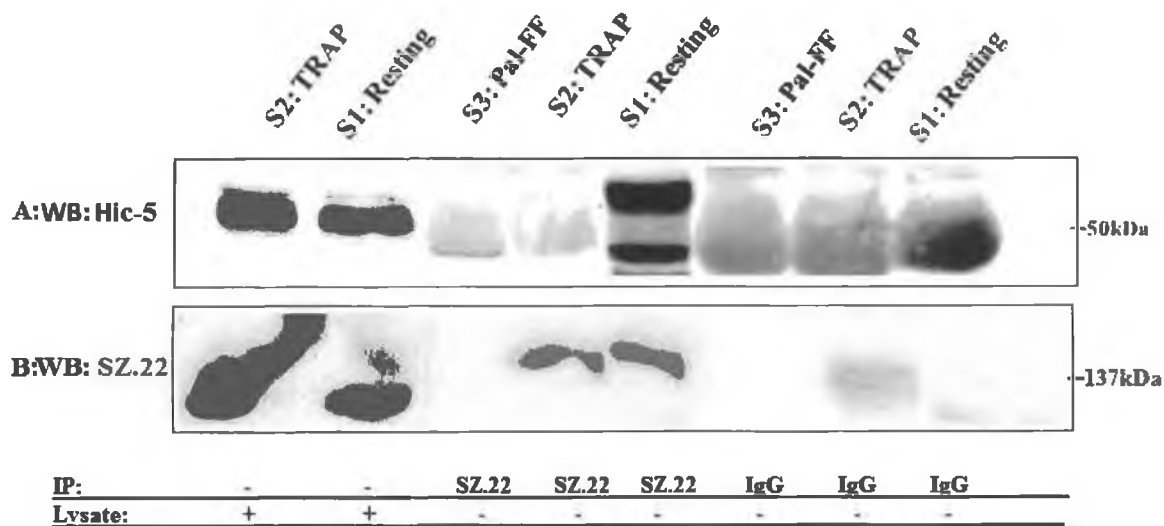


Figure 4.3. Co-immunoprecipitation of SZ.22 antibody with Hic-5 and α_{IIb} proteins.

Washed platelets were left untreated (resting) or were treated with either TRAP (10 μ M) or Pal-FF peptide (100 μ M) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and immunoprecipitated with SZ.22 antibody (α_{IIb} chain of the integrin). Western blot analysis was done using (A) anti-Hic-5 antibody and with (B) anti-SZ.22 antibody. Protein loading concentration is approximately 2.5 μ g. The western blot is representative of 5 independent co-immunoprecipitation experiments.

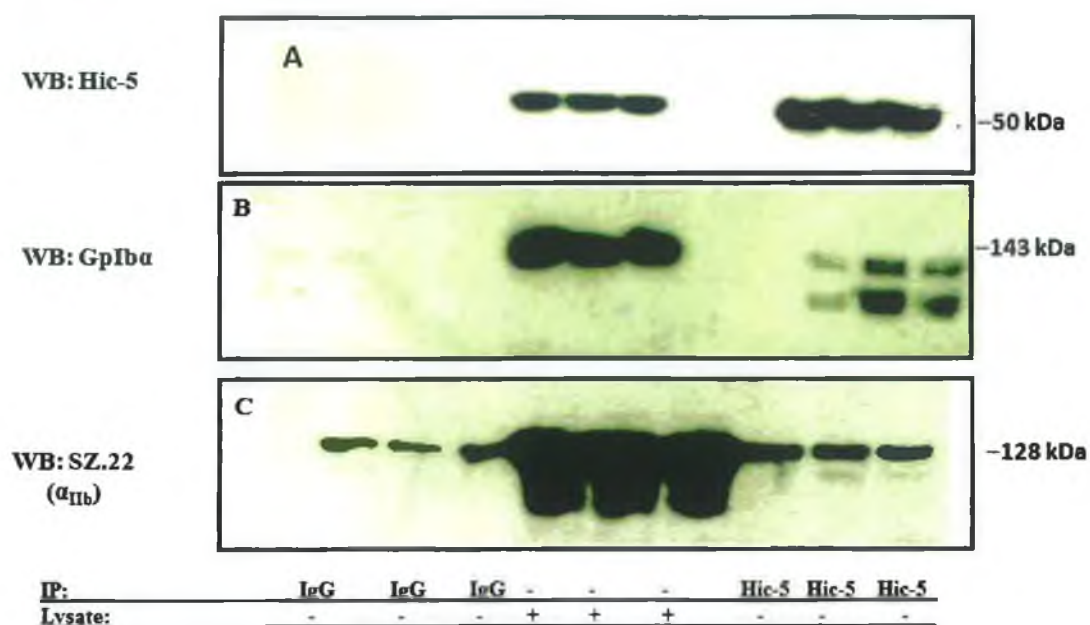
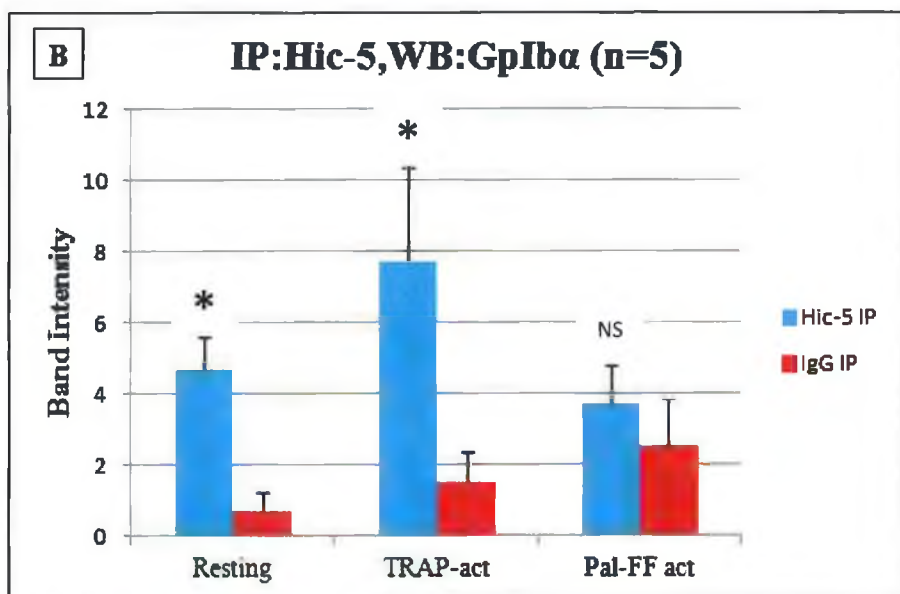
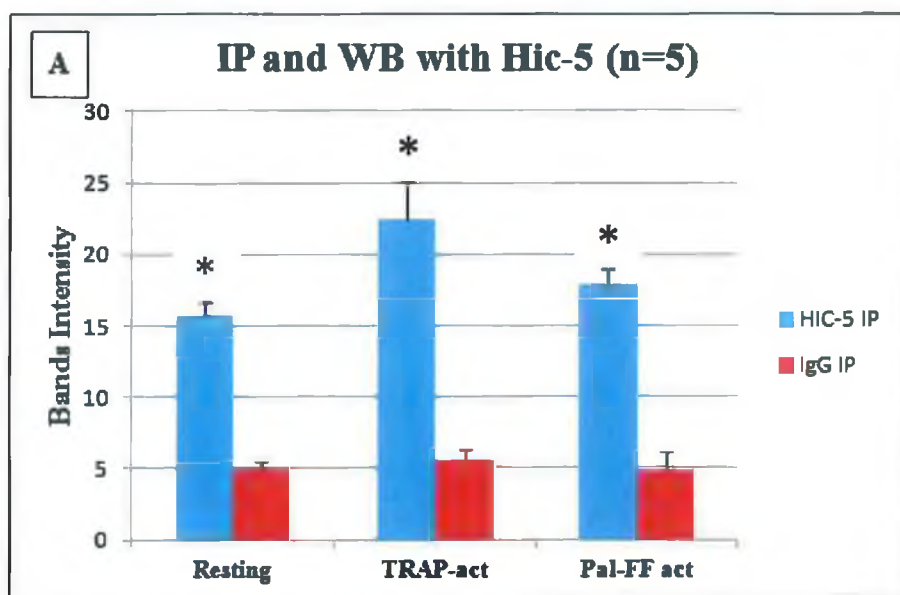


Figure 4.4. Hic-5 antibody selectively immunoprecipitates GpIbα and αIIb

Washed platelets were left untreated (resting) or were treated with either TRAP (10μM) or Pal-FF peptide (100μM) and allowed to aggregate for 3 minutes at 37°C. The samples were lysed and immunoprecipitated with anti-Hic-5 antibody. Western blot analysis was done using (A) anti-Hic-5 antibody, (B) anti-GpIbα antibody and (C) anti-SZ.22 antibody. Protein loading concentration is approximately 2.5μg. The western blot is representative of one of four independent co-immunoprecipitation experiments for SZ.22 and 5 for both Hic-5 and GpIbα. Averaged data is shown in Figure 4.5.



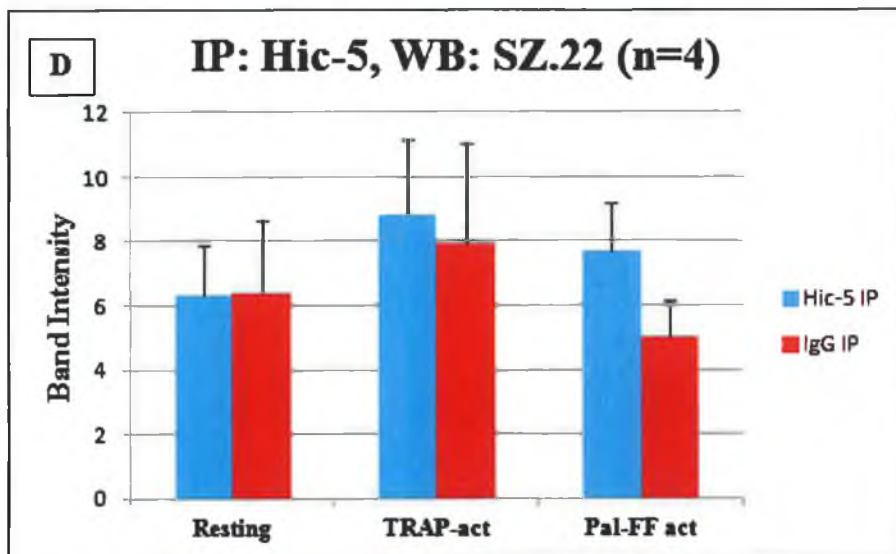
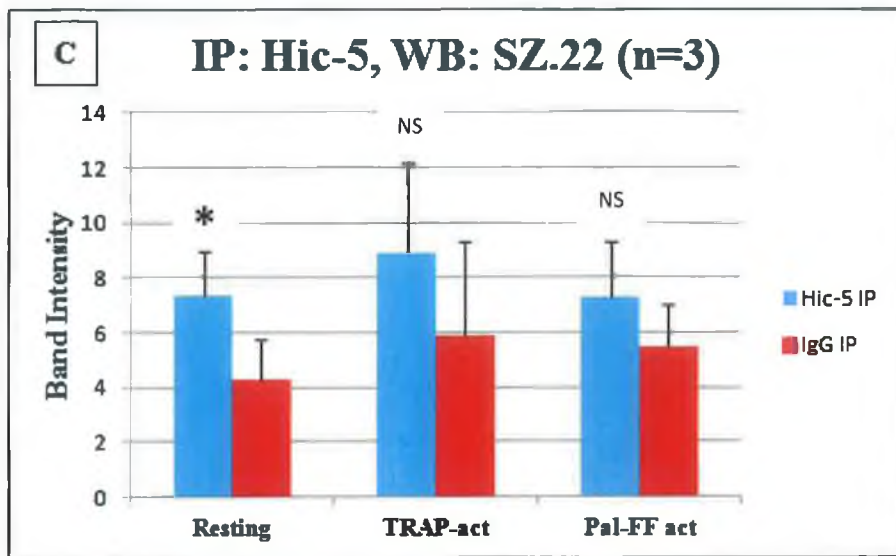


Figure 4.5. Densitometry analysis of proteins' interaction with Hic-5 protein.

The difference in band-intensity between Hic-5 interaction with selective proteins (A) Hic-5, (B) GPIIb α and (C-D) α_{IIb} -subunit and non-specific proteins in lysates. Data are represented as mean \pm SEM for 3 independent experiments. * Indicates $P < 0.05$ compared to IgG control-IP sample, as assessed by two-tailed Student T test. NS= non significant.

4.3 Discussion

In this chapter we have investigated the platelet proteins that bind to the integrin $\alpha_{IIb}\beta_3$ in order to verify data obtained from previous mass-spec analysis of proteins binding to an integrin-derived affinity column (Raab et al., 2010). These experiments had identified known integrin binding proteins (CIB1, Protein phosphatases and RN181) in addition to other uncharacterized platelet proteins. Of these, Hic-5 was the protein most enriched on the peptide column containing the α_{IIb} regulatory motif (KVGFFKR). PINCH-1 and PKAR1 α were also enriched. All three of these proteins were of interest to the RCSI platelet research group. We used the co-immunoprecipitation technique in an attempt to verify and study the interactions between these proteins in platelet lysates.

We successfully immunoprecipitated two proteins using separate antibodies. The first protein was α_{IIb} , using the antibody SZ.22 and the second protein was Hic-5. Subsequent immunoblots using antibodies against the proteins α_{IIb} , PKAR1 α , PINCH-1 and Hic-5 were done to determine if these proteins co-immunoprecipitated with the primary immunoprecipitated protein, α_{IIb} or Hic-5. Densitometry analysis was done to examine the results in a quantitative fashion.

The validity of our experiments is confirmed by additional experiments and controls; we used two different protocols that gave us the same results, and in addition different controls were used.

The antibody anti-SZ.22 specifically pulls down the integrin chain α_{IIb} as expected. Data is presented as representative blots (Figure 4.1A) and by densitometry of 5 independent experiments (Figure 4.2A). There was a significant difference between the proteins that were pulled down with the IgG and the SZ.22 antibodies confirming that the control antibody is selective.

Our co-IP results showed that the proteins PKAR1 α and PINCH-1 do not bind to the integrin α_{IIb} directly in human platelet lysates. In contrast, densitometry analysis of Hic-5 protein showed that this protein does appear to interact with the integrin α_{IIb} .

In parallel experiments, the Hic-5 antibody selectively immunoprecipitated Hic-5 protein, as assumed. By western blotting Hic-5 immunoprecipitates with the same anti-Hic-5 antibody, we observed there was plenty of Hic-5 protein binding to Hic-5 beads and no bands at the IgG

beads, showing that Hic-5 antibody is selective for immunoprecipitation experiments. Densitometry analysis determined the bands intensity of Hic-5 and showed that the pull down bands are significantly higher than the negative control bands in resting and activated platelets (Figure 4.5A).

It was found in a previous study (Arthur et al., 2011) that Hic-5 protein binds directly to GpIb/IX/V. Therefore we did an extra step that confirms these two points. By successfully co-immunoprecipitating GpIb α with the anti-Hic-5 antibody, we confirm that our experimental immunoprecipitation techniques are correct and that Hic-5 antibody is capable of pulling down Hic-5 protein from platelet lysates. The densitometry analysis showed substantial co-association of GpIb and Hic-5 in resting and TRAP activated platelets but not in Pal-FF activated platelets. This raises a question of a role for $\alpha_{IIb}\beta_3$ in facilitating the interaction between GpIb/IX/V and Hic-5, and is consistent with our earlier result demonstrating a potential co-association between integrin α_{IIb} and Hic-5 in resting platelets. Thus Hic-5 protein may cross-link GpIb/IX/V and integrin $\alpha_{IIb}\beta_3$ in resting platelets. Following activation with TRAP, Hic-5 appears to be released from its interaction with α_{IIb} , as suggested in Figure 4.2D, but remains associated with GpIb α (Figure 4.5B). In contrast, activation of platelets with the integrin-derived peptide Pal-FF, destabilizes the integrin α_{IIb} -Hic-5 interaction in a manner that does not permit continued binding of GpIb α to Hic-5.

Hic-5 protein had strong binding signal to the platelet integrin $\alpha_{IIb}\beta_3$ in our proteomics experiments (Raab et al., 2010). As a result, we planned to examine and confirm the integrin co-association with Hic-5 protein. We performed co-IP experiments using Hic-5 antibody and western-blotted for the integrin. In these experiments, we demonstrate that there is a co-association of Hic-5 with α_{IIb} . We observed, in 3 out of 4 experiments resting and activation dependent interaction (in TRAP and Pal-FF activated) platelets (Figure 4.5C). Statistical significance was only observed for proteins from resting platelets in these 3 experiments. However, the 4th experiment changes this overall result. If we include this single outlier result, the interaction in the resting platelet sample is changed. The mean densitometry images shown in (Figure 4.5C) suggest that Hic-5 interacts with integrin α_{IIb} in platelet lysates from resting, TRAP activated and peptide-activated samples.

Nevertheless, there is a high background level of SZ.22 staining observed in the negative control lanes in these experiments, making this conclusion somehow dubious. A number of

methods were explored in an attempt to reduce this background. Such methods included increased numbers and duration of the wash steps performed during the immunoprecipitation, changing the salt concentration in the wash buffers, increasing the amount of detergent in the wash buffers. However, in all cases, we continued to observe high background staining. Thus the results obtained for Hic-5/ α_{IIb} interaction is not clear-cut from these experiments. However, the opposite experimental procedure: immunoprecipitation with SZ.22 followed by western-blotting for Hic-5, clearly demonstrates a co-association of these proteins in resting platelet lysates.

One explanation for the difference in results observed following co-immunoprecipitation of α_{IIb} with Hic-5, and vice versa, is the choice of antibody used. Whereas one antibody was used for SZ.22 immunoprecipitation or α_{IIb} detection, two different antibodies were used in experiments with Hic-5 protein. A rabbit polyclonal antibody was used to probe for Hic-5 in SZ.22-immunoprecipitates whereas a mouse monoclonal antibody was used to immunoprecipitate Hic-5 in experiment. This may be the reason behind the lack of complementarity in the results.

All data is presented as representative images of western blots and the densitometry data was illustrated as charts of n number of separate experiments depending on the protein. Statistical differences were determined using student T-tests.

Conclusion

We have confirmed that the protocols used in our assays are valid. Using these protocols we demonstrated definitively that neither PINCH-1 nor PKAR1 α proteins bind to the integrin α_{IIb} in our co-immunoprecipitation experiments. Finally, we showed that Hic-5 protein interacts to the integrin α_{IIb} but it is possible that this interaction is transient and thus difficult to capture in our experiments.

Confocal Imaging

Work performed by another researcher in the laboratory, using confocal microscopy, lends support to this conclusion. In a series of imaging experiments on $\alpha_{IIb}\beta_3$ -expressing CHO cells adhered to immobilized fibrinogen, it has been demonstrated that talin, a cytoplasmic protein

known to bind to activated integrin, co-localizes with $\alpha_{IIb}\beta_3$ at the periphery of a spreading platelet (Figure 6.1.1), see below. In this experiment, cells are allowed to adhere to immobilized fibrinogen for 45 minutes and are then fixed and stained with two antibodies: CD41 stains $\alpha_{IIb}\beta_3$ and is labelled as red and an α -talin antibody is labelled green. Co-localization is visualized as yellow pixels, that are observed when red and green fluors are co-localized within 10 Angstroms of each other. In Figure 6.1.3, cells are co-stained for integrin (red) and RN181, another known integrin binding protein. Like talin, co-localization was observed at the periphery of the spreading platelet. Next, cells are co-stained for Hic-5 and $\alpha_{IIb}\beta_3$ in Figure 6.1.2. In this image, yellow pixels are observed confirming co-localization of Hic-5 with $\alpha_{IIb}\beta_3$. However, it is notable that this co-localization does not occur at the periphery of the spreading platelets, as was observed for talin and RN181. In contrast, colocalization is only observed in the region of the adhesome, the point where the cell made initial contact with the immobilized fibrinogen. Given that talin and RN181 are known to bind to activated integrin, and Hic-5 may only bind to the resting conformation of integrin in unactivated cells, we then examined the relative staining of cells with RN181 and Hic-5 (Figure 6.1.4). Strikingly, these two protein appear to label alternate pools of $\alpha_{IIb}\beta_3$. We suggest that these pools represent activated and resting integrin, respectively.

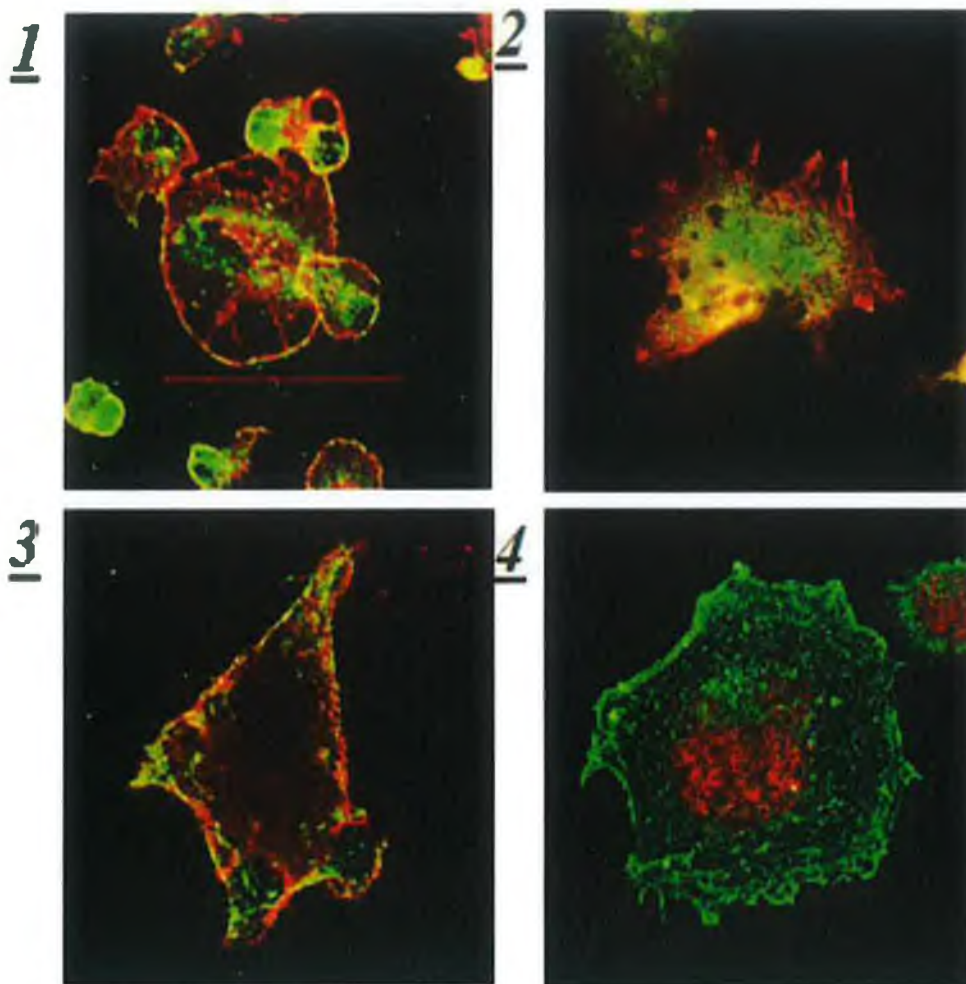


Figure 6.1: Co-localization of $\alpha_{IIb}\beta_3$ integrin with cytoplasmic proteins.

Platelets or CHO-cells expressing $\alpha_{IIb}\beta_3$ were allowed to adhere to fibrinogen-coated glass slides for 45 minutes. Cells were then stained as follows: (1) Talin, a known beta-integrin binding protein is labeled green (FITC) and $\alpha_{IIb}\beta_3$ is stained red (PE). Co-localization is observed as yellow pixels resulting from the juxtaposition of red and green pixels. (2) Hic-5 protein is stained with FITC (Green) and $\alpha_{IIb}\beta_3$ is stained red. Note the localization of Hic-5 in a centralized location but not at the membrane. Co-localization with integrin corresponds to the initial adhesome. (3) RN181 protein is labeled with FITC (Green) and $\alpha_{IIb}\beta_3$ is stained red (PE). Note that RN181 is observed solely at the membrane in these fully spread platelets. (4) Hic-5 protein is stained red and RN181 is stained green. Note the opposite localization of these two alpha-integrin binding proteins. Hic-5 is only observed in the adhesome and RN181 is only observed at the periphery of the cytoplasm. (Images courtesy of Seamus Allen, BSc)

Conclusion: How a scientific knowledge has been advanced by my study?

Western blotting was used to verify the presence of these proteins PKAR1 α , PINCH-1 and Hic-5 in human platelets and to examine their capacity to be altered by platelet activation. We demonstrated that platelets express all 3 proteins. In addition, I show evidence for activation-dependent modification of these proteins in response to pro-thrombotic stimuli. PKAR1 α and Hic-5 show evidence of post-translational modification (phosphorylation or ubiquitination) whereas PINCH-1 shows degradation in response to platelet activation.

We found in a previous study done using proteomics that known integrin binding proteins bind to an affinity column containing the integrin regulatory region, KVGFFKR. This study also identified a range of novel potential integrin-regulating proteins. Therefore, we used Co-IP technique to confirm the interaction of three of these proteins with the platelet integrin. We showed that neither PKAR1 α nor PINCH-1 proteins bind to the integrin chain α_{IIb} . Alternatively, we found that Hic-5 protein probably binds to the integrin. Thus we observe Hic-5 binding to SZ.22-immunoprecipitates from resting platelet lysates. However, we found this result difficult to replicate when we reversed the order of immunoprecipitation and used a Hic-5 antibody as the immunoprecipitating antibody. It is possible that the interaction of Hic-5 and α_{IIb} is transient and thus difficult to capture in our experiments. We found that the proteins PKAR1 α and PINCH-1 belong to protein “W” characteristics, as they do not bind to the integrin at all. In addition, Hic-5 protein shows that it belongs either to protein “X” group when it binds to the resting integrin or belongs to protein “Z”, a protein that binds to the integrin regardless of its activation states.

We have faced a lot of challenges throughout the study that have been an advantage in gaining experimental skills. These challenges have been mentioned in the thesis and include; optimizing experimental procedures for western blotting; using the best type of beads according to the antibodies and handling the beads in co-IP experiments. Finally, we found that using different antibodies types whether they are monoclonal or polyclonal can influence the results.

Moreover, we found that Mass spectrometry analysis of the affinity interacting proteins has a percentage of error and different techniques should probably be used for optimum results. For example, the confocal results shown in chapter 4 can provide powerful support for immunoprecipitation data.

Finally, due to the lack of time we could not increase the number of experiments to confirm the results. Thus, a future work includes more co-IP experiments with Hic-5 and analysis of SZ.22 can be a part of future project.

Appendix

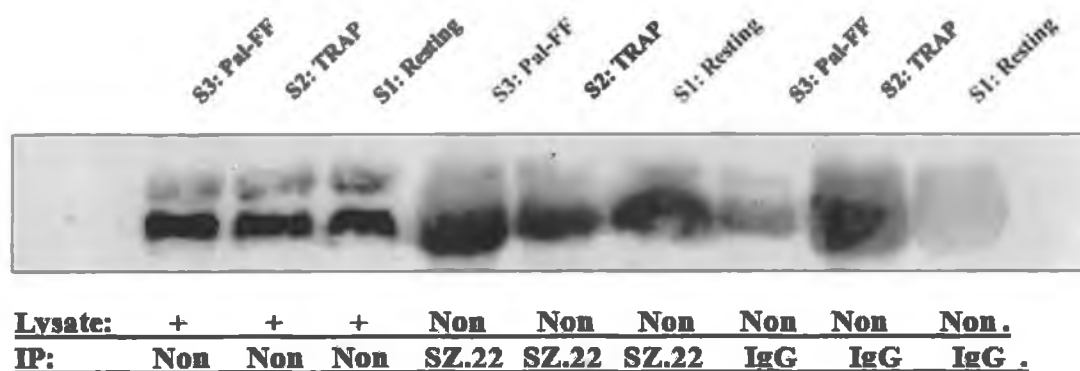


Figure 4.1 (D) was adapted from this western blot. It presents an immunoprecipitation with SZ.22 antibody and western blot with Hic-5(H75) antibody.

Bibliography

The Universal Protein Resource. <http://www.uniprot.org/>.

AIIB, I. SCBT. <http://datasheets.scbt.com/sc-59923.pdf> [Online]. [Accessed].

HIC-5. SCBT-a. <http://datasheets.scbt.com/sc-28748.pdf> [Online]. [Accessed].

HIC-5. SCBT-b. <http://datasheets.scbt.com/sc-137051.pdf> [Online]. [Accessed].

PINCH-1. SCBT. <http://datasheets.scbt.com/sc-47912.pdf> [Online]. [Accessed].

PKAR1A. SCBT-a. <http://datasheets.scbt.com/sc-18800.pdf> [Online]. [Accessed].

PKAR1A. SCBT-b. <http://datasheets.scbt.com/sc-81641.pdf> [Online]. [Accessed].

ARTHUR, J. F., SHEN, Y., GARDINER, E. E., COLEMAN, L., KENNY, D., ANDREWS, R. K. & BERNDT, M. C. 2011. TNF receptor-associated factor 4 (TRAF4) is a novel binding partner of glycoprotein Ib and glycoprotein VI in human platelets. *J Thromb Haemost*, 9, 163-72.

BERNARD, E., PARTHASARATHI, L., CHO, M. K., AYLWARD, K., RAAB, M., DAXECKER, H., O'DUSHLAINE, C. T., SHIELDS, D. C., DEVOCILLE, M., KEYES, T., COSGRAVE, L., O'NEILL, S., MOK, K. H. & MORAN, N. 2009. Ligand switching in cell-permeable peptides: manipulation of the alpha-integrin signature motif. *ACS Chem Biol*, 4, 457-71.

BORDEAUX, J., WELSH, A., AGARWAL, S., KILLIAM, E., BAQUERO, M., HANNA, J., ANAGNOSTOU, V. & RIMM, D. 2010. Antibody validation. *Biotechniques*, 48, 197-209.

BOSSIS, I. & STRATAKIS, C. A. 2004. Minireview: PRKAR1A: normal and abnormal functions. *Endocrinology*, 145, 5452-8.

DAWID, I. B., BREEN, J. J. & TOYAMA, R. 1998. LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet*, 14, 156-62.

DAXECKER, H., RAAB, M., BERNARD, E., DEVOCILLE, M., TREUMANN, A. & MORAN, N. 2008. A peptide affinity column for the identification of integrin alpha IIb-binding proteins. *Anal Biochem*, 374, 203-12.

GUSHIKEN, F. C., PATEL, V., LIU, Y., PRADHAN, S., BERGERON, A. L., PENG, Y. & VIJAYAN, K. V. 2008. Protein phosphatase 2A negatively regulates integrin alpha(IIb)beta(3) signaling. *J Biol Chem*, 283, 12862-9.

HAGMANN, J., GROB, M., WELMAN, A., VAN WILLIGEN, G. & BURGER, M. M. 1998. Recruitment of the LIM protein hic-5 to focal contacts of human platelets. *J Cell Sci*, 111 (Pt 15), 2181-8.

HYNES, R. O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell*, 110, 673-87.

- KATO, A., KAWAMATA, N., TAMAYOSE, K., EGASHIRA, M., MIURA, R., FUJIMURA, T., MURAYAMA, K. & OSHIMI, K. 2002. Ancient ubiquitous protein 1 binds to the conserved membrane-proximal sequence of the cytoplasmic tail of the integrin alpha subunits that plays a crucial role in the inside-out signaling of alpha IIb beta 3. *J Biol Chem*, 277, 28934-41.
- LARKIN, D., MURPHY, D., REILLY, D. F., CAHILL, M., SATTLER, E., HARRIOTT, P., CAHILL, D. J. & MORAN, N. 2004. ICln, a novel integrin alphaIIb beta3-associated protein, functionally regulates platelet activation. *J Biol Chem*, 279, 27286-93.
- LIGNITTO, L., CARLUCCI, A., SEPE, M., STEFAN, E., CUOMO, O., NISTICO, R., SCORZIELLO, A., SAVOIA, C., GARBI, C., ANNUNZIATO, L. & FELICIELLO, A. 2011. Control of PKA stability and signalling by the RING ligase praja2. *Nat Cell Biol*, 13, 412-22.
- LIU, S., THOMAS, S. M., WOODSIDE, D. G., ROSE, D. M., KIOSSES, W. B., PFAFF, M. & GINSBERG, M. H. 1999. Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. *Nature*, 402, 676-81.
- NAIK, U. P., PATEL, P. M. & PARISE, L. V. 1997. Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J Biol Chem*, 272, 4651-4.
- O'NEILL, S., ROBINSON, A., DEERING, A., RYAN, M., FITZGERALD, D. J. & MORAN, N. 2000. The platelet integrin alpha IIb beta 3 has an endogenous thiol isomerase activity. *J Biol Chem*, 275, 36984-90.
- OSADA, M., OHMORI, T., YATOMI, Y., SATOH, K., HOSOGAYA, S. & OZAKI, Y. 2001. Involvement of Hic-5 in platelet activation: integrin alphaIIb beta3-dependent tyrosine phosphorylation and association with proline-rich tyrosine kinase 2. *Biochem J*, 355, 691-7.
- RAAB, M., DAXECKER, H., EDWARDS, R. J., TREUMANN, A., MURPHY, D. & MORAN, N. 2010. Protein interactions with the platelet integrin alpha(IIb) regulatory motif. *Proteomics*, 10, 2790-800.
- RATHORE, V. B., OKADA, M., NEWMAN, P. J. & NEWMAN, D. K. 2007. Paxillin family members function as Csk-binding proteins that regulate Lyn activity in human and murine platelets. *Biochem J*, 403, 275-81.
- STANCHI, F., GRASHOFF, C., NGUEMENI YONGA, C. F., GRALL, D., FASSLER, R. & VAN OBBERGHEN-SCHILLING, E. 2009. Molecular dissection of the ILK-PINCH-parvin triad reveals a fundamental role for the ILK kinase domain in the late stages of focal-adhesion maturation. *J Cell Sci*, 122, 1800-11.
- STEPHENS, G., O'LUANAIGH, N., REILLY, D., HARRIOTT, P., WALKER, B., FITZGERALD, D. & MORAN, N. 1998. A sequence within the cytoplasmic tail of GpIIb independently activates platelet aggregation and thromboxane synthesis. *J Biol Chem*, 273, 20317-22.

- TASKEN, K., SKALHEGG, B. S., SOLBERG, R., ANDERSSON, K. B., TAYLOR, S. S., LEA, T., BLOMHOFF, H. K., JAHNSEN, T. & HANSSON, V. 1993. Novel isozymes of cAMP-dependent protein kinase exist in human cells due to formation of RI alpha-RI beta heterodimeric complexes. *J Biol Chem*, 268, 21276-83.
- VIJAYAN, K. V., LIU, Y., LI, T. T. & BRAY, P. F. 2004. Protein phosphatase 1 associates with the integrin alpha11b subunit and regulates signaling. *J Biol Chem*, 279, 33039-42.



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